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NOVEL TREATMENTS FOR BOTULISM: DEVELOPMENT OF ANTAGONISTS FOR IDENTIFIED STEPS IN THE ACTION OF BOTULINUM NEUROTOXINS

FINAL REPORT

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Using a combination of electrophysiological and biochemical techniques, the action of botulinum neurotoxin (BoNT) was shown conclusively to involve (i) binding/targetting to ecto-acceptors on cholinergic neurons, (ii) acceptor-mediated uptake and, (iii) block of Ca^{2+}-dependent release of transmitters. Domains/chains in BoNT responsible for each step were identified. One useful antagonist of the toxin's binding was developed, in addition to a monoclonal antibody capable of neutralizing the intracellular poisoning. Insights into the molecular action of BoNT were gained, particularly involvement of microtubules in the base of B and, possibly, a large phosphorylated protein concerned with exocytosis. Equivalent experiments on tetanus toxin, and chimeric mixtures of both toxins, yielded valuable information for possible future targetting of agents to cholinergic and non-cholinergic neurons. A more detailed summary is included in the report.

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FOREWORD

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Abbreviations: BoNT A, B, D, E and F, botulinum neurotoxin types; TeTX, tetanus toxin; HC and LC, heavy and light chains; H_2L, BoNT minus the C-terminal half of HC (i.e. H_1); H_2 and $\beta 2$, N-terminal half of HC of BoNT and TeTX, respectively; ACh, acetylcholine; NA, noradrenaline; DTT, dithiothreitol; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; TCA, trichloroacetic acid; PEG, polyethylene glycol; MAPs, microtubule-associated proteins, CCV, clathrin-coated vesicle; CCP, clathrin-coated pit.

INTRODUCTION

Upon embarking on the project, it was known that BoNT type A when applied externally inhibits acetylcholine (ACh) release almost exclusively in the peripheral nervous system (reviewed by Dolly et al., 1986). Accordingly, saturable binding of ^{125}I-labelled BoNT A or B to ecto-acceptors followed by internalization could be observed autoradiographically on cholinergic but not other nerve types (Black and Dolly, 1986, 1986a, 1987; Evans et al., 1988). However, the relevance of such an effective uptake system remained unsubstantiated because there was no direct evidence then for an intra-neuronal action of the toxin, though deductions drawn from pharmacological studies at the neuromuscular junction favoured this (reviewed by Simpson, 1986). Moreover, the latter seemed to underlie the susceptibility of peripheral cholinergic neurons to BoNT since it reduces Ca^{2+}-dependent efflux of ACh and several other transmitters from brain synaptosomes (reviewed by Dolly et al., 1987, 1988), provided high concentrations are employed to overcome the lack of an efficient uptake; also, this could explain the low toxicity of BoNT when applied directly into brain (Williams et al., 1983). Regarding identity of functional domains in the toxin concerned with targetting/internalization, electron-microscopy studies at motor nerve terminals revealed that isolated HC could prevent the binding and subsequent uptake of ^{125}I-BoNT A (Black and Dolly, 1986a), albeit at relatively high concentrations. In contrast, there were reports of HC blocking BoNT binding to cerebrocortical synaptosomes with equal efficacy to the intact molecule (Kozaki, 1979; Williams et al., 1983). Notably, the C-terminal half of HC (H_1 fragment) contributes to this interaction with acceptor sites because the proteolytically-prepared H_2L fragment (intact toxin minus H_1) is unable to antagonise binding of ^{125}I-BoNT A to brain synaptosomes (Shone et al.,

1985). Furthermore, the abilities of HC or its N-terminal moiety (H_2 fragment) to form pores in artificial membranes implicated part of this chain in the uptake step (Donovan and Middlebrook, 1986).

In view of such encouraging findings with these various experimental systems, it was imperative to ascertain if all the toxin fragments exhibit the same pattern of activities on cholinergic nerve terminals and, particularly, whether the binding and uptake phenomena observed in vitro underlie the intoxication. Thus, a neuromuscular junction preparation, the prime target of BoNT, was used to quantify the abilities of the toxin's chains and available fragments (alone and in combination) to block nerve-evoked twitch tension or to antagonise the neuroparalytic effect of the intact toxin. Additionally, the samples were tested on large neurons in Aplysia ganglia because these offered major advantages for investigating the binding, uptake and intracellular action in that toxin could be applied externally and/or internally to cholinergic (or non-cholinergic) cells with electrophysiological recording of evoked quantal release of transmitters. To allow comparative investigations of the toxin's intracellular action in mammalian-derived cells, effects of the toxin preparations on Ca^{2+}-induced release of noradrenaline from cultured pheochromocytoma (PC12) cells were monitored after controlled permeabilisation of the cells with digitonin, under conditions that preserve the exocytosis process. As a more ideal system was desirable, liposomal targetting was developed to allow toxin/fragments to be delivered inside mouse motor nerve endings. A major attraction of these experimental systems was that they could be exploited in assessing anti-toxin monoclonal antibodies being prepared for ability to neutralise the intracellular intoxication step.

With respect to elucidating the toxin's molecular action, there was intense speculation that BoNT enzymatically inactivates its pharmacological target, like some other microbial toxins. As ADP-ribosylations of neuronal proteins were observed by others with impure preparations of type D, a further search was carried out for such activity in homogeneous preparations of BoNT A and B towards nerve terminal components. In particular, a detailed comparison of the latter with type D was conducted; this demonstrated that the supposed enzymatic activity of BoNT D is not related to its inhibition of transmitter release (detailed in midterm report, Ashton et al., 1990). In this context, whilst types A and B lack such ADP-ribosyl transferase activity, it also seemed relevant to establish if the ADP-ribosylated protein occurred in synaptic vesicles where it could function in transmitter release. Finally, due to the definite involvement of phosphorylation/dephosphorylation of neuronal proteins in regulation of transmitter release (Llinas et al., 1985), possible effects of BoNT on the direct phosphorylation in vitro or on the phosphorylated state in situ of proteins in vesicles isolated from rat brain were evaluated.

EXPERIMENTAL PROTOCOLS

Reduction and alkylation of BoNT. Type A BoNT was incubated with 50 mM dithiothreitol (DTT) in 50 mM Tris/150 mM NaCl, pH 8.0 buffer for 60 min at 37°C; the suitability of these conditions for yielding complete cleavage of the inter-chain disulphide was assessed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (PAGE). Native and reduced toxin (as above) were alkylated by treatment with 250 mM iodoacetamide for 15 min at room temperature in the dark. Ellman's reaction was used to determine free sulphydryl content of various BoNT samples treated by (a) alkylation without prior reduc-

tion; (b) reduction without alkylation and (c) alkylation after reduction. For this procedure, BoNT samples (180 µg/sample) were precipitated using trichloracetic acid (TCA) (6.5% final concentration) and resolubilized in 0.8 ml of buffer containing 50 mM Tris-glycine, 1 mM EDTA, 2.6% SDS, pH 8.0. The protein concentration of each sample was determined spectrophotometrically at 280 nm using the established extinction coefficient of 1.47 for a 1 mg/ml solution. Thiols were then assayed by measuring absorbance at 412 nm (ϵ = 14150/M/cm) exactly 10 min after the addition of 30 µl of dithionitrobenzene (stock 5 mg/ml); a blank was treated similarly except 0.8 ml of solubilization buffer was used instead of toxin solution. For competition experiments, the reduced and alkylated toxins were centrifuged to remove any insoluble material and dialysed into a modified Krebs-Ringer medium containing 0.5 mM Ca^{2+} and 5 mM Mg^{2+} (Maisey et al., 1988). Left phrenic-nerve hemi-diaphragms were dissected from Balb C mice and transferred immediately to a closed circulating superfusion system containing 15 ml of aerated Krebs/Ringer solution maintained at 37°C. Nerve-evoked muscle tension was measured against time as described previously (midterm report; Maisey et al., 1988).

Preparation of liposomes entrapping BoNT A LC or HC. The two chains of BoNT A, isolated and characterized as detailed previously (see midterm report and Results section), were renatured by dialysis into 118 mM NaCl/10 mM Hepes, pH 7.4 over 5h (with 3 changes) at 4°C. BoNT was radioiodinated to a high specific activity (≈500 Ci/mmol) as described by Williams et al., 1983; after separation, the iodinated LC and HC gave specific activities of ≈100 Ci/mmol and ≈400 Ci/mmol.respectively. Liposomes were prepared using a modification of a previously described method (Dimitriadis and Butlers, 1979).

Chloroform solutions of phosphatidyl choline/cholesterol/phosphatidyl serine in a 7:2:1 ratio (w/w) were mixed and dried under vacuum. The lipids were suspended in 4ml of the NaCl/Hepes buffer containing LC or HC (0.25 mmol lipid/μmol protein). Trace amounts of the respective iodinated chains were included to allow quantitation of their subsequent entrapment by liposomes. The lipid/protein mixtures were vortexed repeatedly over 30 min at 4°C before sonicating for 1 min with an MSE probe sonicator. After standing for 30 min at 4°C, the mixtures were loaded onto a Sephacryl S200 HR column (2.5 x 20 cm) previously equilibrated in the NaCl/Hepes buffer at 4°C. The liposome peak (6-8 ml) was detected by measuring turbidity ($A_{500\ nm}$) of the fractions collected and the protein quantified by counting radioactivity. The pooled liposome peak was dialysed over 14-16h at 4°C into Krebs-Ringer solution. Samples were aerated (95% O_2/5% CO_2) before being bath applied to nerve-diaphragm preparations for measurement of their effect on nerve-evoked twitch tension.

Preparation of polyclonal and monoclonal antibodies. LC of BoNT A was prepared as described previously except that in preparations used for mice immunisations, an added chromatographic step was employed. Minor contaminating amounts of HC and intact toxin, not readily detectable by silver staining, were adsorbed on a mono-Q Sepharose column equilibrated with 40 mM phosphate buffer, pH 8.4/2 M urea/10 mM DTT. LC was recovered in the void volume and, after dialysis against phosphate buffered saline, showed greatly reduced toxicity (see later).

Two rabbits were immunised repeatedly with 20-25 μg of LC, initially intra-nodally followed at 2-3 weekly intervals with subcutaneous boosts at multiple sites with a similar amount of immunogen mixed 1:1 with Freund's incomplete adjuvant. Maximum antibody

titres were maintained by 3-4 monthly subcutaneous boosts of 20 μg of immunogen. The antibody titre from one of the rabbits (312) was consistently higher and, thus, used preferably in subsequent experiments.

For monoclonal antibody production, Balb/C mice were immunised with 1-5 μg LC either bound to nitrocellulose membranes implanted subcutaneously or injected intra-splenically without the use of adjuvant. This was followed at 2-3 weeks' intervals with 3-4 intra-peritoneal injections with a similar amount of immunogen mixed 1:1 with Freund's incomplete adjuvant. When antibody titres reached 1:1000 to 1:5000, final booster injections of 3-5 μg, without adjuvant, were given twice daily, intravenously and intra-peritoneally respectively, on the 5th, 4th and 3rd day prior to the fusion. The spleen cells were fused with myeloma NS0-1 cells using a modification of the standard polyethylene glycol (PEG) procedure in which the cell suspension was pre-treated with 0.25% PEG and incubated at 37°C for 90 min prior to dropwise addition of 40% PEG. The resulting hybrid cells were selected using Dulbecco's modified Eagle medium containing 1×10^{-4}M hypoxanthine, 4×10^{-7}M aminopterin and 1.6×10^{-5}M thymidine supplemented with 20% foetal calf serum. Antibody production by the hybridomas was screened by ELISA using alkaline phosphatase-labelled goat anti-mouse IgG (and by dot blot analysis). The antibody-producing cells were expanded into 24-wells plates and subsequently into 25 cm^2 tissue cultures flasks and, after preliminary investigations, were selected and cloned by limited dilution. Thereafter, ascites fluids were obtained by injecting intra-peritoneally more than 10^7 hybridomas cells into nude and Balb/C mice which had been primed with incomplete Freund's adjuvant. IgG was purified from the ascitic fluid by affinity chromatography on Protein A Sepharose. The pH of the ascitic fluid was adjusted to

8.0 by the addition of Tris-HCl to a final concentration of 100 mM before being applied onto the resin. After extensive washing of the column with the latter buffer, IgG was eluted with 3 nM KSCN (in 100 mM Tris-HCl; pH 8.0) and dialysed into Tris buffer saline pH 7.4. The subclass of the antibody was determined with the Ouchterlony double immunodiffusion tests using type specific antisera.

Detection of BoNT B-induced changes in synaptosomal phosphorylated proteins. All solutions employed in these experiments contained 0.3 mM phenylmethylsulfonyl fluoride, a protease inhibitor, because certain phosphorylated neuronal proteins are extremely sensitive to proteolysis (see Delorenzo et al., 1979). For the same reason, the cerebral cortex from each individual brain was dissected and homogenised within 45 sec of cervical dislocation of the rat. A P2 preparation of synaptosomes was then made (see Ashton and Dolly, 1988a), resuspended in a physiological buffer and incubated for 90 min with [^{32}P]-Pi (at a concentration of 0.2 mCi/ml) in the presence and absence of BoNT B. These nerve terminals were then washed in Ca^{2+}-free buffer to remove excess toxin and free radioactivity before being exposed to a Ca^{2+}-containing buffer that affords neurotransmitter release, a condition that also produces changes in the phosphorylated state of certain neuronal proteins. After 30s, the reaction was terminated by one of two means. For experiments on intact synaptosomes, a cocktail of kinase/phosphatase inhibitors in 10 mM Mes pH 6.5 was added [the final concentrations (mM) of ingredients were 10, p-nitrophenylphosphate; 20, EDTA; 10, Na, K-tartrate; 25, Na-pyrophosphate; , NaF; 10, Na-orthovanadate] followed by sedimentation of the synaptosomes at 9,000g for 2 min in an Eppendorf centrifuge, prior to electrophoretic analysis. For the detection of changes in phosphorylation of synaptic vesicle and cytosolic pro-

teins, a large amount of radiolabelled synaptosomes (≈8 mg of protein) was mixed 1:1 with a 5-fold dilution of the kinase/phosphatase inhibitor cocktail. These samples were immediately homogenised and the lysed terminals stirred in the normal inhibitor cocktail on ice for 30 min prior to isolation of synaptic vesicles by differential centrifugation (as outlined previously in Ashton et al., 1988b). The final supernatant, representing synaptosomal cytosol, was stored at -80°C; cytosolic proteins were concentrated from this fraction by the addition of TCA (final concentration 10% v/v). The protein precipitate was pelleted, resuspended in TCA, repelleted before two washes in acetone; the latter was removed by evaporation. The final pellets of synaptosomes, synaptic vesicles or TCA-precipitated cytosolic proteins were then solubilised in isoelectric focussing buffer and stored at -80°C. Prior to electrofocussing, solid urea was added to a final concentration of 9.5M together with 2% (w/v) NP-40; 2% (v/v) ampholytes pH 3-10 (2D - Pharmalyte); 5% (v/v) β-mercaptoethanol and 0.15% SDS. These radiolabelled solubilised nerve terminal components were then subjected to 2D-PAGE, by the method of O'Farrell (1975). The focussed rod gels were equilibrated for 15 min in SDS-sample buffer (0.062M Tris-HCl, pH 6.8; 2% SDS; 5% β-mercaptoethanol; 10% glycerol) prior to SDS-PAGE (3-10% gradient gel) in the second dimension. A parallel focussed rod gel was chopped into 0.5 cm sections, each was soaked in 0.5 ml H_2O, and their pH measured to determine the pH gradient. Finally, the gels were fixed, stained with Coomassie Blue, dried and autoradiograms prepared by exposing them to X-ray film with intensifying screens at -80°C. Appropriate protein standards (see figure legend) were run on a separate track in the second dimension gel.

Other procedures. All methods not given here have already been detailed in the midterm report and in our publications cited in figure legends.

RESULTS AND THEIR INTERPRETATION

For ease of understanding, our findings are presented in four sections under headings relating directly to the main goals of the original proposal. As the outcome of experiments determined the precise course of investigations, numerous facets that proved fruitful were pursued intensively - often beyond those specified initially. A few areas of study proposed (eg. isolation and functional characterization of H_1 fragment of BoNT) proved impractical until recently; however, with the advent of new methodologies (eg. H_1 expression) many of these can be undertaken in the new contract. Following the recommended format, outcome of the entire contract effort will be given; such a complete progress report is possible by extensive reference to our submitted midterm report and to published papers (reprints all appended) allowing emphasis to be placed herein on the second eighteen months' work.

Interaction of BoNT and its chains/fragments with neuronal ecto-acceptors; some comparisons with TeTX

For such structure/activity studies, copious amounts of BoNT types A, B, E and F were purified, proteolytically nicked where necessary (eg. B and E) and the two chains from type A and B obtained in homogeneous form, together with H_2L and H_2 from BoNT A, by means of routine procedures established here (Maisey et al., 1988; Poulain et al., 1989a,b; Wadsworth et al., 1990). The latter are detailed in the mid-term report along with the evidence of purity

for each preparation; also, it was shown therein that all of the chains or fragments are relatively non-toxic ($\approx 10^5$-fold less than BoNT) in mice (Dolly et al., 1990), though renaturation of HC and LC together reforms di-chain species and restores much of the toxicity (see later; reviewed in Dolly et al., 1990). For evaluating the functional importance of the toxin's inter-chain disulphide, controlled reduction of BoNT A, and alkylation of its sulphydryls was carried out as detailed in Experimental Protocols.

Differences in the properties of mammalian peripheral and central neuronal ecto-acceptors for BoNT. For investigating the toxins' neuronal ecto-acceptors, rat cerebrocortical synaptosomes are generally used because of the ease with which their saturable binding of ^{125}I-BoNT can be quantified. Our various studies showed that types A, B and F exhibit high and low affinity binding to type-specific sites on synaptosomal membranes; the refolded of each was capable of antagonising binding of the respective partially intact toxin (Williams et al., 1983; Evans et al., 1986; Maisey et al., 1988; Wadsworth et al., 1990). As such interaction of BoNT with brain nerve terminal preparations was not found to lead to toxin uptake nearly as efficient (Black and Dolly, 1987), as noted for peripheral cholinergic nerves (see Introduction), and because of the high content of these acceptors (particularly, the low affinity variety), we suspected that this synaptosomal binding was largely 'non-productive' (Maisey et al., 1988). In order to evaluate this prediction, it was desirable to establish if HC could antagonise the inhibitory action of native BoNT on synaptosomal transmitter release. Unfortunately, these measurements were not very practical because they would have required large amounts of pure HC, due to the relatively high concentrations of the BoNT A needed to give significant block-

ade of synaptosomal transmitter release (Ashton and Dolly, 1988). Thus, attention was turned to the phrenic nerve hemi-diaphragm preparation because of its exquisite toxin sensitivity, with recordings of nerve-evoked muscle twitch tension being adopted (detailed in midterm report) as a convenient assay for BoNT-induced blockade of ACh release. Having established a dose-response curve at 24°C for inhibition of neuromuscular transmission by intact BoNT A and B (Maisey et al., 1988), a comparison of the activity of fragments and chains was carried out. H_2L proved ineffective at 30 nM over 4h, demonstrating that proteolytic removal of H_1 abolishes the neuroparalytic activity (Fig. 1A) and toxicity in mice (Poulain et al., 1989a). It was possible to test fragments for their ability to antagonise the toxin's action using established protocols from the literature for arresting intoxication at the level of toxin binding to the nerve terminals (ie. lowering temperature to 4°C and using elevated Mg^{2+} and decreased Ca^{2+} concentrations). Pre-exposure of the hemi-diaphragm to an excess of H_2L at 4°C prior to adding the intact toxin, failed to alter the BoNT-induced blockade observed after washing the preparation and raising the temperature to 24°C (Fig. 1A). To confirm that this absence of biological activity results from an inability of H_2L to bind to the neuronal acceptors, it was radioiodinated. Whereas ^{125}I-BoNT associated in a saturable manner with motor endplates (identified by ACh esterase staining) in the mouse diaphragm (Poulain et al., 1989b), no such binding of ^{125}I-H_2L was detectable autoradiographically (Fig. 2). As the LC isolated from the same sample of H_2L was toxic inside neurons (see later), it was concluded from these collective measurements that H_1 is essential for targetting/productive binding to motor nerve terminals. Note that removal of H_1 also destroys ability to interact with central synapses because H_2L proved ineffective towards synap-

tosomal transmitter release (Dolly et al., 1990) and was unable to inhibit ^{125}I-BoNT binding to synaptosomes (Shone et al., 1985).

In view of the importance of H_1 for ecto-acceptor recognition in rodents, renatured HC together with LC of BoNT A were tested for ability to block neuromuscular transmission (Maisey et al., 1988 and detailed in midterm report). Whereas each chain alone was without effect (at 30 nM over 4h), a mixture of both did diminish twitch tension but with a 300-fold lower potency than intact BoNT (Fig. 1B). Consistent with the feeble activity of the two chains, excess HC caused no significant reduction in the neuromuscular paralysis produced by native BoNT (Fig. 1C). The most reasonable interpretation of these findings is that for efficient binding/targetting the HC must adopt a conformation equivalent to its structure in the di-chain BoNT molecule. The perturbation of HC structure, by exposure to urea and DTT, during its isolation may not be fully overcome by the renaturation protocol; such subtle differences in conformation would not necessarily be detectable by methods for measuring gross secondary structure parameters (DasGupta, 1989). Experiments outlined below will examine if the inter-chain disulphide plays any part in the toxin-acceptor binding step.

A major implication of the above observations is that the toxin's functional acceptors at the neuromuscular junction differ from their counterparts in brain; however, this deduction is based only on the biochemical properties of the latter. Thus, for a valid comparison to be made an experimental strategy had to be devised that allowed both acceptor types to be studied biochemically; this entailed detergent solubilisation since nerve membranes could not readily be prepared from diaphragm. Triton X-100 extracts of end-plate-containing areas of rat phrenic nerve diaphragm, and cerebrocortical synaptosomal membranes, were prepared and assayed for

saturable binding of 1 nM ^{125}I-BoNT A (blocked by 250 nM unlabelled toxin) by means of a nitrocellulose disc method (Table 1). As a control, specific binding of ^{125}I-α-bungarotoxin was also measured in innervated and non-innervated portions of diaphragm; as expected, a 28-fold enrichment of nicotinic receptors was seen in endplate-containing samples (Wadsworth, 1990). Consistently, the value observed for saturable ^{125}I-BoNT A sites (≈100 fmoles/mg protein under the conditions used) in the nerve terminal-rich sections was 33 times higher than for extra-synaptic regions and similar to that for the synaptosomal extract (Table 1). In both cases, the proteinaceous nature of the acceptors was revealed by their inactivation following treatment of the tissues with trypsin. Importantly, binding to the nerve diaphragm extract was virtually insensitive to neuraminidase treatment but, in contrast, this abolished binding to the central acceptors as we noted previously for synaptosomes (Williams et al., 1983; Evans et al., 1988; Wadsworth et al., 1990). Moreover, the brain acceptors differed in that their BoNT binding was blocked by HC whereas the peripheral neuronal acceptors behaved, as in the functional assay, with BoNT binding remaining unaffected by HC. As reported for synaptosomes, neither H_2L or H_2 gave any change in toxin binding to either extract. Thus, the consistency of results found with both functional and biochemical assays reaffirm that stricter structural requirements do exist for toxin binding to motor n[illegible]ings relative to the situation at central synapses. Indeed, the[illegible]lective though preliminary findings show that the novel acceptor protein identified herein fulfils all the criteria for involvement in toxin targetting/uptake at myo-neural junctions, unlike its counterpart in brain; thus, it is now warranted to establish its molecular and functional properties as proposed in our new contract.

Reduced/alkylated BoNT A antagonises botulinisation of murine nerve terminals. With the di-chain species appearing necessary for productive interaction with acceptor at neuromuscular junction, when relevant concentrations (sub-nanomolar) are used, the possibility arises that HC-sensitive binding of higher BoNT concentrations reported for nerve-muscle preparations (Black and Dolly, 1986; Bandyopadhyay et al., 1987) may represent lower affinity interaction; the latter could mask the more sparse and distinct productive sites. These unexpected findings made the original goal, of obtaining fragments capable of antagonising 'true' toxin-acceptor interaction seem inappropriate until methods (mentioned earlier) became available for isolating H_1 in a biologically-active state. In the meantime, effort was applied to modifying the whole toxin in such a way that it retained ability to bind ecto-acceptors in the peripheral nervous system whilst being non-toxic. Initially, free sulphydryls in native BoNT A were alkylated with iodoacetamide, as demonstrated with the use of Ellman's reagent (see Experimental Protocols). However, such modification gave no detectable change in toxicity in mice and ability to block neuromuscular transmission was retained (Fig. 1D). This surprising finding reveals that the free SH groups are not essential for any steps of the intoxication process. In view of the reports that the inter-chain disulphide was essential for toxicity (Sugiyama et al., 1973), attempts were made to reduce this bond using thioredoxin/thioredoxin reductase, followed by alkylation with iodoacetamide, according to a protocol (Schiavo et al., 1990) found to be readily applicable to TeTX (Fig. 3). Yet, this reduction procedure yielded negligible cleavage of the inter-chain linkage in BoNT A or B as monitored by SDS-PAGE autoradiography and densitometric scanning (Fig. 3), despite using a variety of conditions. As an alternative, a procedure for DTT

reduction of BoNT A was optimised . This gave virtual complete breakage of the inter-chain disulphide according to electrophoretic analysis (Fig. 3) and the subsequent iodoacetamide treatment alkylated all the SH groups (Dolly et al., 1991), as quantified colorimetrically. In the mouse assay, the resultant material showed a 100-fold reduction in toxicity; such a residual level is likely to result from a trace of unmodified toxin. When applied at 2.5 nM to a hemi-diaphragm preparation, the alkylated protein did not alter twitch tension in the time scale of the experiment (Fig. 1D). To gain insight into the basis of this depletion of toxicity, competition experiments were performed, as detailed in Fig. 1A and C, in order to ascertain if the derivative could bind to motor nerve endings. After bath application of 2.5 nM alkylated BoNT A to mouse hemi-diaphragm, at 4°C in modified buffer (to minimize internalization), the incubation was continued in the additional presence of 0.2 nM BoNT A before washing the tissue and raising the temperature to 24°C. Upon neural stimulation, the modified toxin extended the onset and slowed the rate of blockade of muscle tension induced by native BoNT relative to that seen in the control (Fig. 1D). As the experimental conditions employed were designed to reveal interaction at the ecto-acceptor binding step, and taking into account data to be cited later on the intra-neuronal activity of the alkylated material, the observed pattern of antagonism can be attributed to its inhibition of the toxin's productive interaction with the presynaptic membrane, an ability not shared with HC or H_2L (cf. Fig. 1A,C). Clearly, removal of the toxin's inter- and intra-chain disulphides (and/or modification of the resultant SH groups) are without effect on the binding that leads to its uptake and ultimate action. In the case of TeTX, the inter-chain bond was also found to be non-essential for binding but an involvement of the intra-chain

disulphide in HC has not been addressed. Moreover, direct binding to brain membranes of reduced TeTX (by the thioredoxin method) was assessed rather than using a functional assay (Schiavo et al., 1990). Another important outcome of our observed antagonism is that the intoxication has been demonstrated conclusively, for the first time, to involve saturable binding of BoNT to the nerve terminal membrane. Further investigations to be described in a later section will address whether one or other of the two disulphides of BoNT are required for the internalization or intracellular steps. In any case, this available antagonist will be invaluable in future efforts to define the structural properties and physiological roles of the neural acceptors, particularly in distinguishing the 'productive' sites from the bulk population that apparently binds HC.

Structural requirements of BoNT for ecto-acceptor binding in Aplysia neurons exhibits some dissimilarities with mammalian motor nerve endings. Because of these notable dissimilarities in the functional BoNT acceptor at mammalian motor nerve endings and the binding sites characterized biochemically in brain, it was deemed necessary to investigate the productive interaction of BoNT and its chains/fragments with other presynaptic membranes. For this purpose, Aplysia neuro-neuronal synapses were chosen for several reasons (see Introduction; some are also noted later) including the ease with which quantal transmitter release can be recorded electrophysiologically from identified cholinergic and non-cholinergic ganglionic neurons. Bath application of BoNT A (Poulain et al., 1988) or B (Poulain et al., 1990) to the buccal ganglion diminished neurally-evoked ACh release (see midterm report for experimental details). Another attraction of this model is that the toxin's cholinergic specificity resembles the pattern seen in the mammalian peripheral neurons (see

Introduction); externally applied BoNT was very much more effective in inhibiting ACh release than in blocking neurotransmission at a non-cholinergic synapse in the cerebral ganglion of Aplysia (Fig. 4), a feature arising from differences in the neuronal acceptors rather than to disparate intracellular potencies (detailed later). As ACh release could be measured in the neurons at a reduced temperature of 10°C, albeit at a decreased level (Fig. 5A), it was possible to show that BoNT A or E bind under these conditions but were unable to block transmitter release until the temperature was restored to 22°C (Fig. 5B). Note that in the neuron not treated with toxin the initial level of ACh release was resumed when the temperature was switched from 10°C to 22°C. As a similar blockade resulted from a brief (Fig. 5B) or continuous exposure (Fig. 5A) to 10 nM toxin, it can be deduced that rapid binding of each toxin occurred. Furthermore, it is apparent from these results (and other data noted in a later section) that lowering the temperature arrests intoxication after the toxin binding step; hence, this provided an ideal system for investigating the chains or fragments of BoNT involved in the binding (Poulain et al., 1989a,b) that leads to uptake and intracellular action (see sections below). Interestingly, HC was inactive when bath applied at 10°C but prevented the binding of BoNT added later because the blockade of ACh release produced in the control by this short exposure to intact toxin (Fig. 5B) was not recorded (Fig. 5C). Likewise, H_2L or H_2 exhibited similar antagonism of toxin-acceptor interaction (Fig. 5D,E) and demonstrated that H_2 region of HC is predominantly responsible for ecto-acceptor recognition. In fact, H_2 isolated from BoNT A prevented the action of type E (Fig. 5F), highlighting that these two toxins share common binding sites, at least in Aplysia. Notably, these competition experiments established that intoxication of this preparation also

involves saturable binding of BoNT, but differs in that only H_2 is involved, whereas the dichain molecule is required at the rodent neuromuscular junction. Notwithstanding this greater structural stringency for BoNT binding to rat motor nerve endings, it is likely that the role of H_2 documented for Aplysia applies also to mammals; there it could act in conjunction with H_1 (domain in intact BoNT) which was shown earlier to be essential in both peripheral and central neurons.

Distinct ecto-acceptors underlie the characteristic neuronal specificities of BoNT and TeTX. With the aim of further characterizing BoNT acceptors, the Aplysia model was exploited to compare their neuron specificities with that of TeTX (Poulain et al., 1991a). Upon bath application to cholinergic neurons in the buccal ganglion, the dose-response curves obtained for inhibition of neurotransmission showed BoNT A to be ≈100-fold more potent than TeTX (Fig. 4A). In contrast, the reverse situation was observed with non-cholinergic neurons, though in this case only the time courses for fixed toxin concentration were tested (Poulain et al., 1991b) because of the greater difficulty of quantifying quantal release at these terminals. Such distinct preferential actions arise from these neurons possessing the requisite cell surface acceptors for these toxins rather than dissimilar intracellular efficacies (see below). Although it is notable that the preference of BoNT for cholinergic neurons accords with that in murine peripheral nerves (see Introduction), the different specificity of TeTX cannot be defined because of the unknown identity of the transmitter operative at the non-cholinergic terminal of Aplysia studied. However, the latter is an excitatory synapse whereas TeTX blocks predominantly transmitter release at inhibitory junctions in vertebrates.

LC of BoNT and TeTX are active when placed inside motor nerve endings and other mammalian cells: a vestigial role for HC of BoNT in Aplysia neurons

Initial efforts were made to demonstrate an intra-neuronal site of BoNT action by applying the protein to the cut end of motor axons leading to motor endplates in mouse diaphragm; lack of alteration in subsequently recorded nerve-evoked muscle tension indicated that this technique was unsuccessful as a means for internalizing the active toxin. Likewise, incorporation of BoNT inside resealing synaptosomes (after the nerve terminals are pinched off during homogenisation of cerebral cortex) was not efficient enough to give inhibition of K^+-stimulated transmitter release. Yet, both these methods have been refuted to allow proteins access to the nerve terminal interior. At that juncture, Aplysia neurons were used to allow micro-injection of toxin into identified neurons, together with recording of quantal transmitter release as before.

The chains of BoNT and TeTX required inside Aplysia neurons for blockade or transmitter release. In this system, BoNT A was equally active (Fig. 4B,D) inside cholinergic (Poulain et al., 1988, 1988a; Maisey et al., 1988) and non-cholinergic neurons (Poulain et al., 1991a); notably, this was also true for TeTX and, in fact, both displayed similar potencies (Poulain et al., 1991b). Thus, an intracellular site of action was demonstrated conclusively for these two related toxins, a finding confirmed using permeabilised PC12 cells (McInnes and Dolly, 1990) and chromaffin cells (covered in mid-term report). Digitonin-permeabilised PC12 cells produced Ca^{2+}-dependent exocytosis of [^{3}H]noradrenaline (NA) and this was inhibited effectively, though incompletely, by BoNT A (Fig. 6A). As these toxins, when placed intracellularly, block Ca^{2+}-dependent

release of all transmitters tested both in Aplysia and rat brain synaptosomes (Ashton and Dolly, 1988; reviewed in the mid-term report), as well as inhibiting exocytosis from endocrine cells, their targets are ubiquitous component(s) of this fundamental process. Also, it is clear that the toxins' distinct neuronal specificities result from selective targetting/uptake mediated by acceptors present on the susceptible cells.

Attention was next focussed on the chains/fragments of these toxins that act intracellularly; for this purpose, the convenient Aplysia model was first used. Micro-injection of BoNT LC (Fig. 7A) into a cholinergic neuron of the buccal ganglion did not alter quantal transmitter relase unless HC was also applied intraneuronally (Poulain et al., 1988; mid-term report) or added to the bath (Fig. 7A). HC alone was inactive within the cell (shown in a later Fig.). Obviously, the intracellular presence of both chains of BoNT A or B (Poulain et al., 1990) is required for blockade of ACh release; the same was found for a non-cholinergic neuron with LC alone being inactive unless co-administered with HC (Fig. 8A,B). Internal application of H_2L (or H_2 plus LC) also proved ineffective until HC was added (Fig. 7B) so it emerged that H_1 was the region required for LC to be active (detailed in mid-term report). In this regard, it is interesting that HC of TeTX could not substitute in this role for its counterpart from BoNT in cholinergic cells (Fig. 7C). A further difference exists between the toxins in that LC alone of TeTX, applied internally, blocked release from cholinergic (Fig. 9) or non-cholinergic neurons (Fig. 8A,B); its HC was ineffective (Fig. 9).

HC is not required within mammalian cells (unlike Aplysia neurons) for LC to inhibit exocytosis. This curious situation of both chains

of BoNT being essential inside Aplysia neurons whilst LC of TeTX was adequate created the need for further investigations on another cell type. The system already established for measuring release from permeabilised PC12 cells was used initially. Here, LC alone of BoNT was equally effective as intact toxin in decreasing Ca^{2+}-elicited [^{3}H]NA release (Fig. 6). In contrast, HC on its own proved ineffective and it had no significant effect on the action of LC (Fig. 6B). Similar findings have been reported for LC of TeTX and BoNT in both PC12 and chromaffin cells (reviewed Dolly, 1991). In this context, it is relevant that pre-treatment of BoNT A with 10 mM DTT did not potentiate its blockade, consistent with the minimal reduction of the inter-chain disulphide that occurs under the conditions used (McInnes and Dolly, 1990).

At this stage, it was unclear if the latter results for BoNT chains seen with permeabilised cultured endocrine cells (not truly representative of neurons) or the different findings observed with quantal transmitter release from Aplysia neurons were typical of the rapid, neurally-evoked ACh release that occurs at mammalian neuromuscular junction, the prime target of BoNT. For this reason, new methodology was developed to allow each of the toxin's chains to be delivered via liposomes inside nerve endings of mouse hemi-diaphragm (de Paiva and Dolly, 1990); this is detailed in Experimental Protocols. After sonicating a lipid mixture in the presence of LC from BoNT A, an acceptable level of this moiety was found to be incorporated into the resultant liposomes, isolated in the void volume of a gel filtration column (Fig. 10B). Negatively-charged lipids were employed to minimize toxin chains associating with the vesicles rather than being entrapped; thus, when LC was added to preformed liposomes a negligible amount of protein co-eluted with the vesicles (Fig. 10A). On the other hand, a quantity of HC did associate with

preformed liposomes (Fig. 10C) due to its hydrophobic nature; nevertheless, a large amount of HC seemed to become entrapped because of the increased levels seen when added to the lipids prior to sonication (Fig. 10D). Application of such liposomes to the phrenic nerve hemi-diaphragm at 37°C in Krebs solution resulted in their fusion with nerve and muscle membranes and delivery of the contents, as established by quantitation of uptake of an inert radioactive tracer into endplate-enriched and non-endplate areas of diaphragm. When LC-containing liposomes were used, a time- and concentration-dependent diminution of neuromuscular transmission occurred (Fig. 11A). Such an effect was not seen with liposomal encapsulated HC (Fig. 11B) or control liposomes devoid of toxin and made with the buffer used in the twitch experiments (Fig. 11A); likewise, a contribution from any contaminating intact toxin was excluded (de Paiva and Dolly, 1990). Evidence for a presynaptic inhibition of ACh release was provided by the temporary, though incomplete reversal caused by 4-aminopyridine, a blocker of certain voltage-activated K^+ channels in the nerve membrane that facilitates transmitter release as a result of increasing Ca^{2+} influx. To evaluate if HC could influence the activity of LC, a mixture of two separate preparations of liposomes containing LC and HC, respectively, was also added. An identical lag period and paralysis-time was observed for this mixture and the LC-containing liposomes; the slight difference in the shapes of the curves falls within experimental error. Notwithstanding the limitation of this 'mixing' protocol, HC appears to be unnecessary for toxicity. Because bath application of LC (at the same concentration as above) in the absence of liposomes failed to affect synaptic transmission, it is concluded that liposomes deliver LC inside mammalian motor nerve terminals where it mimics the toxin's action. This represents a major advance because it consolidates the

proposed scheme of intoxication and, also, establishes that LC can be used as a safer and more selective probe for future studies on its intracellular target.

In the light of these findings, the involvement of HC in the toxin's action inside Aplysia neurons, a feature lost through evolution in mammals and, for the action of TeTX in all cells examined, would seem to be secondary. For example, it could be speculated that interaction of HC (probably via its hydrophobic H_1 domain) with the toxins' intracellular target is a prerequisite for recognition of LC. This scenario is more likely than HC associating with LC because HC (processed type A) can enable single-chain BoNT E (or H_2L) to block ACh release in Aplysia neurons (Poulain et al., 1989b).

Production and characterization of antibodies against the intraneuronally active chain of BoNT. In view of the documented activity of BoNT LC within these several cell systems, and in order to reach the initial goal of obtaining agents capable of neutralising the intoxication at an intracellular stage, polyclonal and monoclonal antibodies raised were raised against this chain. As it was highly desirable for this purpose to immunize animals with 'native' LC (ie. not a toxoid derivative), it was necessary to isolate this protein to an extremely high level of purity (<4 x 10^3 mouse LD_{50}/mg) by removing on a mono-Q Sepharose column (see Experimental Protocols) any intact toxin or HC contaminating the LC

, purified by published procedures. After extensive experimentation, immunisation regimes were devised for rabbits and mice that avoided death of the animals and yielded antibodies with the appropriate properties. In ELISA (detailed in Experimental Protocols), the resultant rabbit antisera showed high titres towards BoNT A LC or intact toxin (1:30,000 dilution) but no cross-reactivity was

detectable with HC of types A or B, or BoNT B, E, or F (Fig. 12). Consistently, on immunoblots the antiserum reacted with free LC and only the latter in the whole toxin (Fig. 13C). Assays in mice showed a neutralisation capacity of >2.5 x 10^5 LD_{50} units/ml for antiserum 312 (higher dilutions are being used to determine the absolute value). In preliminary experiments intracellular injection of either serum into cholinergic neurons in buccal ganglion of Aplysia prevented the inhibitory action of bath-applied BoNT A (2 nM) on ACh release. Although these encouraging results make further characterization of the antibodies worthwhile in the future, effort has been concentrated on isolating monoclonal antibodies against LC because of their greater usefulness in the longer-term.

Of the several anti-LC monoclonals selected initially, one (G5)-IgG 2b isotype-looks particularly valuable. An IgG concentrate of this monoclonal was obtained from the resultant ascitic fluids and its properties are shown. On ELISA, it reacted at 1:32,000 dilution with LC or intact BoNT A whilst being unreactive with HC or types B, E, F or TeTX (Fig. 12). Likewise, immunoblotting of SDS-PAGE gels demonstrated its exclusive reaction with LC of type A (Fig. 13A,B); moreover, radio-immunoprecipitation using ^{125}I-BoNT A confirmed that it recognises LC in the intact di-chain protein. Although G5 was ineffective in the mouse neutralisation tests, when the hybridoma supernatant was air-pressure injected into a cholinergic neuron of Aplysia (≈1000-fold final dilution) it prevented the action of bath-applied 15 nM BoNT A (Fig. 14). As two presynaptic neurons synapse onto the same postsynaptic cell in this preparation, it was possible to inject a control hybridoma supernatant into the other cell; this was totally ineffective and demonstrated that the neutralisation seen was indeed due to anti-LC IgG. The latter is a very exciting advance because it illustrates how intracellularly

neutralising monoclonals, suitable for future neuronal targetting (outlined in our new contract), can be prepared, and exemplifies how available experimental models are applicable in their evaluation. Additionally, the liposomal delivery system will be applied to test the effectiveness of such antibodies inside vertebrate motor nerve endings.

N-Terminal portion of HC from BoNT or TeTX can inter-changeably internalize either LC into Aplysia neurons but uptake at the mammalian neuromuscular junction requires the dichain form of BoNT

Because only intact BoNT is active at murine endplates, investigations were conducted on Aplysia neurons to gain insight into the chains/domains concerned with the uptake step because the N-terminal moiety of HC, H_2, is capable of ecto-acceptor binding (see earlier section). It was readily established that LC alone cannot be internalized; its bath application (at high concentration) to a neuron in the buccal ganglion pre-injected with HC was without effect on ACh release (Fig. 15A). This also shows that HC when placed inside the neuron cannot mediate the uptake of LC added extra-cellularly. In contrast to LC, application of HC to a cell previously injected with LC diminished ACh release (Fig. 7A), showing that HC can internalize on its own. Furthermore, HC is able to mediate uptake of LC when present together in the bath (Fig. 15C) or subsequent to washing (Fig. 15B), confirming results of earlier described competition experiments (Fig. 5C) that it binds saturably (Maisey et al., 1988). Notice that HC of type B brings about uptake of LC of A or B. Unlike HC, prior addition of LC followed by washing before inclusion of HC is ineffective (Fig. 15D), indicating the inability of LC to bind or remain attached to the neuronal membrane for sufficient time to interact with the other chain. Just as removal of H_1 did not

affect the binding of H_2L (Fig. 5D,F), likewise, this did not alter internalization because bath application of H_2L to a neuron pre-injected with HC (to provide H_1, which is essential inside) reduces ACh release (Fig. 15E). Further experiments revealed that the separated H_2 could enable LC to enter; intra-neuronal administration of HC, followed by LC inclusion in the bath gave no change in transmitter release until H_2 was added (Fig. 15F).

When the internalization of TeTX chains were examined in Aplysia, the same results were obtained. LC alone could not enter neurons (Poulain et al., 1990) but HC (Fig. 16A,B) or β_2 (Poulain et al., 1991a) mediated its uptake. Thus, the N-terminal halves of HC in both BoNT and TeTX can bring about acceptor-mediated internalization in Aplysia neurons (cf. the contrasting situation at neuromuscular junction). Considering the distinct neuronal specificities exhibited by BoNT and TeTX for cholinergic and non-cholinergic synapses in Aplysia (see above), and in view of the ability of HC or N-terminal portion from each toxin to mediate internalization of their respective LC, we addressed the intruiging question of whether this chain/fragment from BoNT could internalize LC of TeTX and vice versa, thereby switching the toxins' characteristic neuron selectivities. In the first set of experiments on cholinergic cells, bath application of the constituent chains of TeTX proved much less effective in blocking ACh release than their counterparts from BoNT (Fig. 16A), consistent with the disparate potencies of the intact toxins noted earlier. Interestingly, upon substituting the TeTX HC with that from BoNT, TeTX LC was made appreciably more potent in reducing ACh release (Fig. 16C). In fact, the equivalent result was achieved using H_2 of BoNT and LC of TeTX in the bath (Fig. 16E). Corresponding experiments on non-cholinergic neurons demonstrated that the extracellular application of the chains of TeTX were far

more powerful in blocking transmitter release than the equivalent addition of HC and LC of BoNT (Fig. 16B), again in accord with results for the intact proteins. Use of HC of TeTX enabled LC of BoNT to be internalized effectively into the non-cholinergic neuron because this mixture gave a dramatic reduction of transmitter release (Fig. 16D) relative to that seen with both chains of BoNT (Fig. 16B), provided HC of BoNT was pre-injected to provide H_1. Such an enhancement of the activity of BoNT LC was also achieved with β_2 fragment of TeTX (Fig. 16F). Hence, this very convincing series of novel results, showing that the chimeric mixtures displayed potencies equivalent to those of the respective parent toxins whence the HC originated (Poulain et al. 1991b), illustrate the ability of N-terminal halves of each HC to efficiently internalize LC from either toxin. Furthermore, it is clear that their binding to distinct ecto-acceptors on the two neuron types determines the toxins' specificity because BoNT and TeTX exhibit similar potencies intracellularly. If it can be assumed that BoNT and TeTX can act, also, with similar potencies inside mammalian neurons, their preferential action in the peripheral and central nervous systems, respectively, would be ascribable to their selective uptake at these sites. Based on these collective findings in Aplysia, it may be postulated that H_2 or β_2 possess two functional domains, one being distinct and responsible for the divergent neuronal specificity, whereas the other serves a common role in translocating LC of either toxin.

In the light of these observations, it is now relevant in the current contract to establish the nature of the interaction between HC (or indeed N-terminal portion) and LC that allows internalization to take place in Aplysia neurones. Already, initial investigations have revealed that separately refolded HC and LC of BoNT can associ-

ate under conditions, used in Aplysia experiments, to form a disulphide-linked dichain species (Dolly et al., 1991). Moreover, preliminary studies on reduced/alkylation BoNT A (detailed earlier) using the Aplysia system show that in addition to being able to bind ecto-acceptors efficiently, this derivative is active intracellularly. Thus, it is tempting to postulate that an inter-chain disulphide bridge may be essential for internalization both in Aplysia and mammalian motor nerve endings, as was proposed for TeTX where this bond was exclusively reduced with thioredoxin/thioredoxin reductase (Schiavo et al., 1990). In this case, certain features may be shared by the uptake system in vertebrates and invertebrates; this may include H_2 being concerned with binding/uptake at neuromuscular junction but requiring the essential contribution of H_1.

Clues to the molecular actions of BoNT A and B

Effects of altering intra-synaptosomal levels of second messengers on the action of BoNT A and B. To validate the use of synaptosomes as a convenient preparation for studies on BoNT action, we examined the effectiveness of elevating Ca^{2+} concentration on the reversibility of blockade of transmitter release caused by type A and B. Increased extracellular levels of Ca^{2+} were ineffective in antagonising the inhibition of K^+-stimulated NA release from cerebrocortical synaptosomes induced by BoNT A or B unless the Ca^{2+} ionophore A23187, was included (Fig. 17A,B). Interestingly, this treatment caused a greater extent of reversal of the intoxication seen with type A than B, reminiscent of findings at motor nerve terminals when using 4-aminopyridine (Hansel et al., 1987), reaffirming some subtle difference in their mode of action (reviewed in Dolly, 1990). Ca^{2+}-induced changes in protein kinase C activity do not appear to underlie this reversal because incubation of poisoned synaptosomes

with an active phorbol ester, phorbol 12-myristate 13-acetate (an activator of the enzyme) was unable to mimic the effect of Ca^{2+} plus ionophore (Fig. 17C,D). The inhibitory effects of the toxins also remained unaltered when the synaptosomes were pre-treated with 8-bromo c-GMP or dibutyrl c-AMP, in the presence of a phosphodiesterase inhibitor (Fig. 18 A,B). Likewise, exposure of intoxicated nerve terminals to 8-bromo c-GMP or nitroprusside (a stimulator of guanyl cyclase) failed to perturb the BoNT-induced reduction in evoked transmitter release (Fig. 18C,D).

Possible involvement of the cytoskeleton in BoNT intoxication. Because of the implication of cytoskeletal elements in transmitter release (Lindstedt and Kelly, 1987), the influence of disassembling the actin- and tubulin-based cytoskeleton on the toxin's action was examined. Breakdown of microfilaments in synaptosomes with cytochalasin D did not affect the subsequent blockade of NA release by BoNT A or B (Ashton and Dolly, 1991). In contrast, depolymerization of microtubules with colchicine attenuated the inhibition of transmitter release normally produced by BoNT B whilst that of type A remained unchanged (Fig. 19A,B). The significance and specificity of this treatment were confirmed by the same pattern of results being observed with two other microtubule-dissociating drugs, nocodazole and griseofulvin (Fig. 19C-F). As these agents have dissimilar structures it seems unlikely that such an interesting finding could have resulted from secondary effects of all these drugs; moreover, the noted restriction of this antagonism to type B toxin accords with known differences in the precise sites of action of BoNT A and B. Notably, complete protection against the detrimental effect of BoNT B was not achievable even when using a combination of the drugs. Also, it must be emphasised that, under the conditions

employed above (where drugs were removed during washing stages), K^+-evoked Ca^{2+}-dependent transmitter release was not altered by these agents alone. However, if colchicine was present throughout the experiment the amount of release was reduced (see legend to Fig. 20) but these conditions produced no greater antagonism of BoNT B intoxication (Fig. 20A). Further evidence for the involvement of microtubules was gained from the observation that stabilisation of this cytoskeletal component by treatment of synaptosomes with taxol attenuated the effect of colchicine on the intoxication caused by BoNT B (Fig. 20B); treatment of synaptosomes with taxol alone produced no change in [^{3}H]NA release. Evidence available to date indicates that colchicine does not affect the measurable binding of ^{125}I-BoNT B to synaptosomes (Ashton and Dolly, 1991), or internalization of BoNT B into synaptosomes, leading to the suggestion that the drug perturbs the toxin's intra-neuronal action. Although there is no direct biochemical assay for measuring the influence of colchicine on BoNT internalization, it can be assessed indirectly by exploiting the steep temperature-dependence of the final stage of the intoxication. After exposure to synaptosomes for 90 min at 30°C, BoNT B should have bound and become internalized; yet, it exerted a minimal effect (20% inhibition) on NA release. However, removal of extracellular toxin by washing and re-incubation of the nerve terminals for a further 90 min at 37°C resulted in release being inhibited to the normal extent. With such a double incubation protocol, microtubule-disrupting drugs can be added either during the first time period (when binding and internalization occurs) or during the second phase (when the toxin exhibits its intracellular action). An antagonism of BoNT B action is seen only when colchicine is added during the latter step (Ashton and Dolly, 1991); therefore, this drug must act at a stage after the binding and internali-

zation of the toxin. Findings with BoNT A support this suggestion; colchicine did not inhibit ^{125}I-BoNT A binding or its internalization.

It has still to be demonstrated whether BoNT B acts directly on the microtubule system or whether its breakdown simply activates certain other processes which counteract the ability of the BoNT B to block transmitter release. For example, depolymerization of microtubules leads to an increase in the fluidity of the plasma membrane (Aszalos et al., 1985), thereby leading to the activation of various enzymes, including adenylate cyclase. However, to date no enzyme system that can be activated by this cytoskeletal rearrangement, has been found to antagonise the action of BoNT B. Enzymes tested include adenyl cyclase, guanyl cyclase and protein kinase C. An explanation for the incomplete inhibition of toxin action by colchicine (and similar acting drugs) is that there are certain microtubules that are insensitive to these agents (Gordon-Weeks et al., 1982), and that BoNT B exerts its effect upon both populations. In view of these results, it is pertinent to consider the proposed involvement of cytoskeleton in the release of transmitters and hormones (Lindstedt and Kelly, 1987). The actin-containing elements are believed to form a barrier which prevents vesicle/granule fusion (Cheek and Burgoyne, 1986; Lelkes et al., 1986) whereas the tubulin-based cytoskeleton may play a role in the transport of vesicles to release sites (reviewed by Walker and Agoston, 1987), though no direct involvement of microtubules in neurotransmitter release has been documented. One speculative interpretation of the data presented here is that BoNT B prevents the normal detachment of synaptic vesicles from microtubules and, hence, transmitter release is reduced. Recently, these results with type B BoNT have been confirmed on other neurotransmitter terminals

and preliminary data indicate that the action of TeTX can also be antagonised by these microtubule-disrupting agents, lending more credence to this hypothesis. Possibly, type A may also act on microtubules but only on the colchicine-insensitive variety. As various proteins have been suggested to be concerned with attaching vesicles to microtubules, these would seem likely candidates for the toxin's target. One such protein, synapsin I, can be excluded because it is absent from adrenal medullary chromaffin cells (De Camilli and Greengard, 1986) even though BoNT blocks exocytosis from these cells (Stecher et al., 1989).

BoNT increases phosphorylation of a high M_r protein in nerve terminals. In initial experiments, BoNT B intoxication appeared to produce an increase in the phosphorylation of a high M_r protein in synaptic vesicles. This radiolabelled band was very near the top of 5-20% polyacrylamide gels and, thus, assigned a tentative M_r of ≈280 kDa. In order to determine its size more accurately and to establish if it is related to any microtubule-associated proteins (MAPs), 3-10% gradient gels and immunoblotting techniques were employed. No phosphorylated protein corresponded to those detected by antibodies to MAP-1 or MAP-2 and, furthermore, the toxin-sensitive radiolabelled moiety appeared to have an M_r of ≈180kDa in this refined gel system. On two-dimensional SDS-PAGE, the labelled protein gave a pI ≈5.0 (cf. Fig. 21). Repetition of these experiments several times indicated that the exact amount of radiolabelled protein varied between experiments, suggesting that this phosphorylated component is very labile. Also, it became apparent that BoNT A treatment of nerve terminals induced a change in phosphorylation of this 180 kDa protein, although to a lesser extent than that produced by BoNT B.

In order to compare, under identical conditions, the phosphorylation of proteins of synaptic vesicles with those of intact synaptosomes, the latter were prepared for gel analysis by having their phosphorylated state 'frozen' using a kinase/phosphatase inhibitor cocktail (see Experimental Protocols). The latter was found to yield a higher amount of phosphate incorporation into proteins relative to that seen upon stopping phosphorylation with SDS-gel sample buffer (as normally used). Under these conditions synaptosomes also showed a 180 kDa M_r protein with a pI ≈5.0 whose phosphorylated state was increased by BoNT B (Fig. 21A) or A (not shown), relative to non-toxin treated control (Fig. 21B). No phosphorylated spot corresponding to this protein was seen in toxin-treated or control cytosol, reaffirming that it is a membraneous component. However, its content is low both in synaptosomes (predominantly plasma membranes) and vesicles since it was not detectable in Coomassie blue stained gels of samples of these membranes. Relative to the total complement of phosphorylated proteins, there appeared to be a similar amount of this radiolabelled polypeptide in each fraction, although these values are very difficult to quantitate. Thus, it would appear that this labelled protein is not particularly enriched in the vesicles.

At this moment, the two best candidates for the identity of this protein are both found associated with clathrin-coated vesicles (CCVs) from neurons. AP180/AP3 (Ahle and Ungewickell, 1986; Prasad and Lippoldt, 1988; Keen and Black, 1986) is believed to be an assembly peptide for clathrin insertion into clathrin-coated pits (CCPs); it has virtually the exact same pI and M_r as the phosphorylated protein detected herein. The phosphorylation state of AP180/AP3 (a major substrate of CCV kinases) has been speculated to be involved in the formation/regulation of CCPs/CCVs (Morris et al.,

1990). As these are found at the plasma membrane, AP180/AP3 should also reside therein if synonymous with our protein; to date, it has been detected in [^{32}P]-labelled intact neurons (Keen and Black, 1986). Intriguingly, another neuronal specific protein associated with CCPs, NP185 (Kohtz and Puszkin, 1988) is also similar to our speculative toxin substrate, although its pI (≈ 6.0) is slightly higher (a criteria Kohtz and Puszkin use to state that NP185 is not the same as AP180/AP3). This protein probably associates with other subcellular organelles since there is an excess of this over clathrin. An association of NP185 with tubulin has been shown (Kohtz and Puszkin, 1989), and this cytoskeletal component (or one of its isoforms) may control the binding of this protein to CCVs.

It will be very worthwhile establishing if one or more of these proteins partake in botulinisation, particularly since CCVs are involved in endocytosis. Perhaps, the synaptic vesicles which secrete much of the neurotransmitter (the readily releasable pool) undergo many exocytotic/endocytotic cycles (cf. Trimble et al., 1991) and that BoNT prevents endocytosis and therefore eventually exocytosis, by changing the phosphorylated state of such a protein. Electron micrographs indicate that there is no depletion of synaptic vesicle number in botulinised preparations, but this could mean that the readily releasable pool of vesicles (particularly those docked at the active zone) represents only a small fraction of the total number present. An alternative explanation is that, as a consequence of BoNT inhibiting exocytosis, there is a concurrent reduction of vesicle membrane retrieval by endocytosis, and it is this that leads to the increase of the phosphorylated protein ie. it is simply a secondary effect of the toxin.

CONCLUSIONS

In the duration of this contract, all 4 main objectives were achieved in large part. Further to the progress detailed in the midterm report, accomplishments include:

(i) Validating experimental protocols for measuring membrane-bound ecto-acceptors responsible for productive recognition of BoNT A (or TeTX) in Aplysia neurons and rodent motor nerve terminals.

(ii) Establishing that cholinergic and non-cholinergic cells in the sea-snail possess distinct binding sites with which H_2 (of types A and E) or β_2 interact, respectively, creating their characteristic susceptibilities to BoNT and TeTX.

(iii) Provision of evidence that functional BoNT A ecto-acceptors on rat phrenic nerve membranes differ biochemically from the binding components on synaptosomes, previously described in the literature, particularly that the former only recognises di-chain toxin, whereas the latter binds HC.

(iv) Preparation of an effective antagonist of BoNT, by controlled reduction and alkylation, that binds effectively at murine motor nerve endings but is not internalized (at least in Aplysia; unpublished observations).

(v) Demonstration that H_2 and β_2 are responsible for acceptor-mediated uptake of LC (alone cannot gain entry) from BoNT or TeTX into Aplysia neurons, with neuron specificity of chimeric mixtures being determined by the respective fragment used.

(vi) Showing that LC alone of BoNT acts intracellularly in mammalian motor nerves and PC-12 cells whilst HC (or H_1 by inference) is also required inside invertebrate neurons even though LC of TeTX is active in all these systems.

(vii) Production of anti-LC monoclonal antibody shown to neutralise intra-neuronally the inhibitory action of BoNT A on ACh release from

Aplysia neurons.

(viii) Exclusion of cyclic nucleotides, protein kinase C and ADP-ribosyl transferase (Ashton et al., 1988a; Ashton et al., 1990) in the toxin's action though small G-proteins of unknown function were found on purified synaptic vesicles (see midterm report; Matsuoka and Dolly, 1990).

(ix) Discovering that disassembly of synaptosomal microtubules with several drugs antagonises the inhibition by BoNT B of transmitter release (but not A), whereas this effect is counteracted by stabilisation of this cytoskeletal component with taxol.

(x) Observing a high M_r synaptosomal protein whose phosphorylation is increased during intoxication with BoNT.

Implications of these numerous findings for future research are:

(a) Having conclusively documented the triphasic intoxication process, and largely identified the toxin chain or domains responsible for each step, development of antagonists for the latter has now become a reality. Progress towards this end will be greatly facilitated by the various experimental strategies already devised to study independently each phase of the poisoning.

(b) Having found an antagonist (reduced/alkylated BoNT) that binds to functional acceptors at mammalian neuromuscular junctions, but seems unable to efficiently enter the nerve terminal, provides an invaluable tool for future studies. For example, it can be used in functional competition experiments to ascertain if the various types of BoNT truly interact with distinct 'productive' ecto-acceptors, a question very pertinent to many facets of the toxin research programme. Addditionally, this novel probe will enable the physiological role of BoNT acceptors to be assessed by allowing the effect of their occupancy (without the associated pronounced toxicity of

native BoNT) to be measured in various neuronal preparations, as proposed for the new contract. Such an approach may, also, yield some insight into the endogenous ligands for these acceptors. Finally, a possible longer-term application of the r luced/alkylated derivative could be its development as an antagonist for clincial evaluation; this would entail modifying selected residues in the LC portion that are essential for intracellular action, thereby excluding any toxicity even if some of the material became internalized *via* a non-specific route.

(c) A valuable outcome of these studies in *Aplysia* is that the structural restrictions of the neuronal uptake system for BoNT seems less rigid than suspected since HC or H_2L can enter alone or they can take in LC; also, H_2 is able to internalize the LC. Similar results were seen with the corresponding chain/fragments of TeTX and, more import ntly, chimeric mixtures of LC and HC or H_2/β_2 yielded toxicity with the neuronal specificity being determined by the requisite N-terminal portion of HC used. Although the uptake system at mouse motor nerves exhibits more stringent structural requirements, the above-mentioned findings highlight the possible scope for altering LC or attaching adducts (eg. antibody FAB fragments) without loss of internalization; indeed, as a consequence of the *Aplysia* experiments, HC of BoNT has been used in conjunction with LC of TeTX to achieve a more effective block of neuromuscular transmission by the latter, albeit with a potency lower than that of native BoNT (Weller *et al.*, 1991). Collectively, this pattern of results strengthens the highly sought-after prospect for neuronal targetting of BoNT-neutralising antibodies, or indeed other therapeutic agents.

(d) Availability of a monoclonal anti-LC antibody capable of neutralising the neuroparalytic action of relatively high amounts of BoNT

A, internalized in the normal way (ie. recognised after any changes that occurred upon uptake), together with the progress noted in (c) raises the likelihood of accomplishing in the future an effective treatment for botulism.

(e) Steady progress with pinpointing the target for BoNT B is encouraging, particularly the involvement of microtubule-based cytoskeleton, together with the initial characterization of a large synaptosomal protein that becomes phosphorylated during the intoxication. Further pursuit of such investigations, as well as studies on the substrate for type A (as proposed in the new contract), are of the greatest importance because of the vital part played by these ubiquitous components in Ca^{2+}-dependent release of transmitters in all systems tested.

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APPENDIX

Figure Legends

Fig. 1. The effect of intact, chemically modified, and fragments of BoNT type A upon neuromuscular transmission of the mouse phrenic nerve hemi-diaphragm preparation. Muscle twitch, evoked by stimulating the phrenic nerve (0.2 Hz square waves of 0.1 ms duration and 1.5V amplitude), was recorded by means of a transducer. In A, B and D, H_2L (△), HC (•), LC (0) or reduced/alkylated (R/A) BoNT (◇) were found to be without effect. An equi-molar mixture of both chains (B,▲) gave a blockade equivalent to that seen with a 300-fold lower concentration of BoNT (B,□). Alkylated, but not reduced BoNT (D,▲) proved to be as active as the native toxin (D, 0). In A, C and D, the tissues were preincubated at 4°C in the absence (open symbols) or presence of H_2L (A, ●), HC (C, ■, ●) or reduced/alkylated BoNT (D, ●) for 30 min in a buffer (containing 0.5 mM Ca^{2+} and 5 mM Mg^{2+}) designed to minimize their internalization. After simultaneous incubation with 0.3 nM BoNT for 15 min (A, C) or 30 min (C) or with 0.2 nM BoNT for 15 min (D), the hemi-diaphragms were washed extensively in the latter buffer and then in normal physiological medium before raising the temperature to 24°C, initiating stimulation and monitoring the muscle tension. Adapted from Poulain et al., (1989b); Maisey et al., (1988) and Dolly et al., (1991).

Fig. 2. Interaction of ^{125}I-BoNT and ^{125}I-H_2L with mouse motor nerve terminals. Purified BoNT and H_2L were radioiodinated to high specific radioactivity (1000-1700 Ci/mmol) and assessed for their ability to bind saturably to mouse phrenic nerve hemi-diaphragm preparations. Following incubation of the tissues with the labelled derivative (see below), longitudinal sections (10 μm) were cut and stained for ACh esterase to reveal motor endplates. Subsequently, standard autoradiographic techniques were used to visualise the radioactive labelling. A and B, sections treated with 1 nM ^{125}I-BoNT A in the absence (A) or presence (B) of 1 μM unlabelled BoNT A for 90 min at 22°C followed by extensive washing. Note the heavy clustering of silver grains (in A) localised on the motor endplates (arrow) which was prevented by an excess of the unlabelled toxin (B). C, sample labelled with 1 nM ^{125}I-H_2L as in A. The absence of silver grains on the motor endplates, shows the inability of this fragment to recognise the toxin's acceptors. D, a control specimen not treated with radioactivity but showing ACh esterase stained endplates. Taken from Poulain et al. (1989b).

Fig. 3. Reduction of native BoNT A and TeTX with DTT or thioredoxin/thioredoxin reductase. The reduction of inter-chain disulphide was determined by running the samples on SDS-PAGE (under non-reducing conditions), staining the gel with Coomassie blue, and quantitating the amount of intact toxin relative to separated chains by densitometric scanning of the gel tracks. (A) BoNT A was reduced with DTT and the reaction stopped by adding excess iodoacetamide. (B) 0.2 mM NADPH and 2 μM thioredoxin in 100 mM sodium phosphate/1mM EDTA buffer, pH 7.5 was added to 0.5 mg/ml TeTX or BoNT maintained at 4°C. After adddition of 0.1 μM thioredoxin reductase, the temperature was raised to 30°C. After 60 min the reaction was stopped with 0.5 mM iodoacetamide. Note that the inter-chain disulphide of BoNT is virtually unaffected whilst that of TeTX is reduced.

Fig. 4. Relative potencies of BoNT and TeTX in blocking transmitter release at cholinergic and non-cholinergic synapses of Aplysia when applied extra- and intra-neuronally. In A and B, BoNT or TeTX was either bath applied (A) to the buccal ganglion for 30 min or air-pressure injected into the presynaptic cell body (B). In each case, the time taken for a 50% reduction in neurally-evoked ACh release was determined (by recording the amplitude of the postsynaptic response) and plotted against the extracellular concentration of toxin (A) or the calculated intra-somatic concentration (B). An equivalent set of measurements of non-cholinergic transmission was made (C,D) using the cerebral ganglion after bath application (C) of either toxin for the time shown (hatched area) or following intra-neuronal administration (D) of each (arrow). In these experiments, the time courses of blockade of transmitter release were monitored and the average points plotted, after making allowance for the extent of run-down of the postsynaptic response seen in control samples. A,B adapted from Poulain et al. (1991a) and C,D from Dolly (1991).

Fig. 5. Effect of cooling and HC, H_2L or H_2 on the inhibition of neuronal ACh release induced by BoNT A or E in Aplysia neurons. In all experiments, the amplitude of postsynaptic responses elicited by a presynaptic action potential was measured. Control values were recorded at 22°C, and the preparation was then cooled to 10°C for the time identified by arrows (A). After stabilisation of postsynaptic responses at a reduced level, BoNT A (10 nM) was bath-applied (horizontal lines), producing no effect during the subsequent 2h period. On restoring the temperature to 22°C, a rapid decrease of response amplitude ensued, indicating a blockade of synaptic transmission. (B) shows the same experiment as in (A) except that, after 10 min of application (short horizontal lines), BoNT A (●) or E (○) (10 nM at 10°C) was removed by washing for 45 min. Upon re-equilibration at 22°C, there was a decrease in response amplitude. Using the ability shown in (A) and (B) of reduced temperature to arrest BoNT poisoning at the membrane binding step, the ability of HC, H_2L or H_2 to compete for interaction with membrane ecto-acceptors was assessed. 200 nM HC, H_2L or H_2 (C, D or E) was added to the bath (hatched area) for 30 min; BoNT A (10 nM) was then bath-applied for 10 min (horizontal lines), followed by extensive washing. In a similar experiment (F), H_2L was also found to counteract the reduction of ACh release normally caused by nicked BoNT E (10 nM). Adapted from Poulain et al. (1989b).

Fig. 6. Reduction of NA release from permeabilised PC12 cells by BoNT or its chains. Cells were loaded with [^{3}H]NA, washed, treated with digitonin and then incubated with toxin or its various fragments. Release of [^{3}H]NA was then evoked with 10 μM free Ca^{2+}, and Ca^{2+}-dependent secretion determined after subtraction of release in the absence of this cation. The results are expressed as a % of the non-toxin treated control. (A) Dose-dependence of the inhibition by BoNT A (○) or its LC (●). (B) The effect of DTT on the inhibitory action of BoNT and the influence of HC on the potency of LC. Adapted from McInnes and Dolly (1990).

Fig. 7. Inability of internally-applied LC or H_2L to block ACh release from Aplysia neurons unless HC of BoNT (but not TeTX) was present. ACh release was evoked in the buccal ganglion by a presynaptic action potential and the postsynaptic response that ensued recorded as a percentage of the control against time. (A) The LC from H_2L (at a calculated intracellular concentration of ≈20 nM) was

injected (arrow) into the presynaptic neuron without modification of ACh release. A decrease in ACh release was observed only upon addition to the bath (hatched area) of the HC (40 nM). (B) Intracellular injection (arrow) of H_2L into the presynaptic cell (calculated intracellular concentration >10 nM) did not change evoked ACh release. Subsequent addition to the bath (hatched area) of the HC (40 nM), which enters the cell, inhibited transmission. Inserts represent recordings of action potentials and postsynaptic responses (upper of each insert) 1h 40 min after injection of H_2L and 2h after bath addition of the HC (right hand side); note that in the latter the postsynaptic response is decreased whilst the presynaptic action potentials remains unchanged. Calibration: vertical, 475 nS (response) and 50 mV (action potential); horizontal, 100 ms. (C) A combination of HC of TeTX and LC of BoNT injected intra-neuronally (each at a final intracellular concentration ≈20 nM) failed to induce a blockade of ACh release. A,B adapted from Poulain et al. 1989a; C from Poulain et al. (1991a).

Fig. 8. Dissimilar domains of BoNT A and TeTX are responsible for their intracellular inhibition of transmitter release at a non-cholinergic synapse. After control recordings were made, evoked postsynaptic responses were monitored following the intracellular injection of LC_{Te} or LC_{Bo}, each at a final calculated intra-somatic concentration of ≈100 nM (A), or of an equimolar mixture of HC_{Bo} and LC_{Bo} (intra-somatic concentration of ≈20 nM) (B). Taken from Poulain et al., (1991a).

Fig. 9. Intracellular effects of isolated chains of TeTX. After control recordings, HC or LC of TeTX were injected into a presynaptic cholinergic neurone (arrow., final intracellular concentration ≈200 nM). A rapid decrease in postsynaptic response ensued injection of LC but not HC. Recordings of the action potential and of postsynaptic response (upper of each recording) are shown at the times indicated. Note that whilst postsynaptic response was suppressed, no change in action potential was detectable. Calibrations: vertical, 300 nS (response) and 40 mV (action potential); horizontal, 65 ms. Taken from Poulain et al., (1990).

Fig. 10. Gel filtration of LC and HC of BoNT encapsulated in or associated with liposomes. Liposomes prepared as described in Experimental Protocols, together with proteins and radioiodinated tracers, were loaded onto a Sephacryl S200 HR column and eluted at a flow rate of 1 ml/min; 1.5 ml fractions were collected. The liposome and protein peaks were detected by determining the absorbance at 600 nm and radioactivity of each fraction. (A,C), elution of preformed liposomes that were incubated for 30 min at 4°C with LC (A) or HC (C) prior to application to the column. (B,D), elution of liposomes containing LC (B) or HC (D) prepared by sonication of the lipids in the presence of the chains. Adapted from de Paiva and Dolly (1990).

Fig. 11. Effect of control liposomes and those containing BoNT HC or LC on neuromuscular transmission. Liposomes in aerated Krebs solution were bath applied to mouse phrenic nerve-hemidiaphragm preparations which were superfused at 37°C and stimulated supramaximally. Nerve-evoked muscle tension, expressed as a % of the initial valve was measured against time. Control liposomes, lacking protein (▲,○) failed to produce any change as did liposomes entrapping HC (■,●). Liposomes encapsulating LC at final concentrations of 20 nM (△), ≈15 nM (□) and ≈9 nM (◇) decreased muscle tension.

In B, preparations of liposomes containing LC were halved and simultaneously bath applied to nerve-diaphragm preparations with either a three-fold excess of liposome entrapped HC (◆) or liposomes containing no protein (▲). The data presented for the LC and HC liposomes were obtained with different chain preparations. A is adapted from de Paiva and Dolly (1990).

Fig. 12. Titration curves for monoclonal and polyclonal antibodies to BoNT A LC as determined by ELISA. Purified toxins (500 ng), and LC or HC (100 ng), were coated onto wells of microtitre plates (Immulon IV) and ELISA performed with varying dilutions of rabbit polyclonal and mouse monoclonal antibodies. Reaction of $BoNT_A$ (0) and LC_A (●) with the monoclonal; $BoNT_A$ (△) and LC_A (▲) titres with rabbit polyclonal antibodies. (□), Lack of cross-reactivity of HC_A, HC_B, subtypes B, E or F with both the monoclonal and polyclonal antibodies. Note that with all of the latter no cross-reactivity was seen, even though 2.5 µg of toxin was used.

Fig. 13. Specificity of monoclonal and polyclonal antibodies for BoNT A LC. (A) Silver-stained SDS-PAGE of samples of BoNT types A, B, E, F and TeTX (0.5 µg protein/track). (B) An equivalent gel was blotted onto nitrocellulose and probed with the purified anti-LC A monoclonal antibody, followed by detection with a second antibody-alkaline phosphatase conjugate. (C) SDS-PAGE and immunoblot of BoNT A and LC (tracks 1, 2 and 3, 4 respectively) visualised by silver stain (tracks 1 and 3) or anti-LC rabbit polyclonal antibody (1:500 dilution) as described above (tracks 2 and 4).

Fig. 14. A monoclonal antibody to LC of BoNT A neutralises intraneuronally the toxin's inhibition of ACh release. Supernatant of the hybridoma (G5) was air-pressure injected into the cell body of a presynaptic neuron in the buccal ganglion of Aplysia whilst into a second cell, that synapses onto the same postsynaptic neuron as the latter, an equivalent sample from an unrelated hybridoma was administered similarly. Evoked release of transmitter was quantified by recording the postsynaptic response, as detailed elsewhere. Neither injection caused any change in ACh release but when 15 nM BoNT A was bath-applied the previously-administered monoclonal antibody prevented the subsequent inhibition of neurotransmission (open squares) induced by toxin in the control neuron (closed squares).

Fig. 15. Effect of sequential application of HC, LC or fragments of BoNT on ACh release in Aplysia neurons. In all experiments, ACh release was evoked in the buccal ganglion of Aplysia by a presynaptic action potential and amplitude of the responses was recorded in the postsynaptic voltage-clamped neuron. These were expressed as a percentage of the control values and plotted as a function of time. (A) HC of BoNT A was injected (arrow) into the neuron followed by bath application of 100 nM LC of BoNT A (hatched area). (B) HC of BoNT B (40 nM, first hatched area) was bath applied for 1h and this was without effect on release. After free HC was removed by extensive washing (75 min), the addition of LC of type B (40 nM, second hatched area) to the bath resulted in a blockade of release. (C) Successive application of LC type A (10 nM, first hatched area) and of HC type B (10 nM, second hatched area) into the extracellular medium induced an irreversible decrease in ACh release. (D) Bath application of LC of BoNT A (first hatched area) followed, after extensive washing, by addition to the bath of HC did not affect the response. (E) Addition of 40 nM H_2L to the bath (hatched area) which, alone, did not affect ACh release (see neuron o) induced a

decrease in ACh release when the presynaptic neuron (●) had been pre-injected with HC (arrow, calculated intracellular concentration >10 nM. (F) Changes in the postsynaptic response resulting from the presynaptic stimulation of a neuron (●) injected with HC (arrow, intracellular concentration ≈10 nM) were compared with those of a second, non-injected (○) presynaptic cell, afferent to the same postsynaptic neuron. After bath application of LC (40 nM, hatched area), addition to the bath (dashed area) of H_2 (40 nM) induced a depression of neurotransmitter release only in the cell injected with HC. A,D taken from Poulain et al. (1990); B,C adapted from Maisey et al. (1988) and E,F, are from Poulain et al. (1989a).

Fig. 16. Effects of hybrid mixtures of the chains and fragments of BoNT and TeTX on neurotransmitter release at cholinergic and non-cholinergic synapses in Aplysia. These experiments were performed as outlined in Fig. 4 except mixtures of the individually renatured HC and LC of the two separate toxins (A,B), and combinations of the two toxin's chains (C,D) or fragments therefrom (E,F) were bath applied (hatched area). In (D) and (F), HC of BoNT was injected intra-neuronally. Note that HC or H_2 of BoNT made LC of TeTX more effective in blocking ACh release (C); similarly, HC or B_2 of TeTX made LC of BoNT (when HC of BoNT was present inside) more effective in non-cholinergic cells (D). Adapted from Poulain et al. (1991a) and Dolly (1991).

Fig. 17. The influence of A23187 and phorbol myristate acetate on BoNT-induced inhibition of Ca^{2+}-dependent K^+-evoked release of [^{3}H]NA from synaptosomes. Samples were incubated at 37°C for 90 min in the absence (○, ●) or presence of BoNT A (□, ■, ◇, ◆) or B (△, ▲, ▽, ▼), washed and transmitter release measured over a 5 min period following the addition of buffer containing 25 mM K^+ (final concentration). In A and B, 200 nM BoNT was employed. A, [^{3}H]NA release observed with different Ca^{2+} concentrations (○, □, △) or the latter plus 30 μM A23187 (●, ■, ▲) dissolved in dimethylformamide: ethanol [3:1 (v/v)]; an equivalent amount of the solvent was included in all samples. B, Transmitter efflux seen with 1.2 mM Ca^{2+} plus various concentrations of A23187 in the absence (●) or presence of BoNT A (■) or B (▲). Note that batches of A23187 varied in their potency; for example, when another lot was dissolved in dimethylsulfoxide, 1-10 μM gave a similar effect to those shown. As the ionophore did not increase the amount of lactate dehydrogenase present in the supernatant of treated synaptosomes, its effects are not attributable to Ca^{2+}-dependent lysis. In C, D, various BoNT concentrations were employed with (◇,▽) or without (◆,▼) a 6 min treatment with 1 μM phorbol myristate acetate, before release was stimulated. The resultant values in C and D are expressed relative to that for the non-toxin treated control. Taken from Dolly et al. (1990).

Fig. 18. Increasing the intra-synaptosomal levels of cyclic nucleotides does not alter the dose dependency of the blockade of transmitter release by BoNT A or B. Synaptosomes were incubated for 30 min at 37°C in buffer alone (■) and with 1 mM isobutylmethylxanthine (IBMX) plus 0.1 mM 8-bromo c-GMP (A,○) or 1 mM IBMX plus 1 mM db-cAMP (B,□) before incubation with BoNT B for 90 min at 37°C. After washing, K^+-evoked [^{3}H]NA release was then measured over a 5 min period. In another series of experiments, synaptosomes were incubated for 90 min at 37°C in the absence (control) or presence of BoNT B (C) or A (D) prior to incubation for 15 min at 25°C with 0.1 mM 8-bromo c-GMP (C,●) or 1 mM nitroprusside (D,△) or buffer alone

(■), followed by stimulation of release. The amounts of Ca^{2+}-independent [^{3}H]NA efflux observed have been subtracted from the total; the resultant values are expressed relative to that for the non-toxin treated control. Data shown represent the mean of 2 independent experiments; error bars show the range. From Ashton and Dolly (1991).

Fig. 19. Microtubule dissociating agents antagonise the inhibitory action of BoNT B (but not Al on Ca^{2+}-dependent K^+-evoked [^{3}H]NA release from synaptosomes Rat cerebrocortical synaptosomes were incubated at 37°C for 30 min with 1 mM colchicine (A,B), 10 μg/ml nocodazole (C,D) or 200 μg/ml griseofulvin (E). Various concentrations of BoNT were then added and incubation continued for 90 min. Control samples were treated similarly except for the omission of the microtubule drugs (■). Synaptosomes were subsequently washed and Ca^{2+}-dependent, K^+-evoked [^{3}H]NA release was measured over a 5 min period. The observed amounts of Ca^{2+}-independent [^{3}H]NA efflux have been subtracted from the total: resultant values are expressed relative to that for the non-toxin treated control. The values presented for release are the mean for 3 (± SD) (B), or 2 (± range) for (C,D,E,F). For A the number of independent experiments for each value is indicated by the number shown; the significance of difference between colchicine-treated and non-treated samples is indicated by asterisks above the paired points (*, 10%; **, 5%; ***, 2%; ****, 1%). From Dolly (1991) adapted from Ashton and Dolly (1991).

Fig. 20. Antagonism by colchicine of BoNT B-induced blockade of NA release and its reversal by taxol. In (A) synaptosomes were incubated at 37°C for 30 min with (●) or without (■) 1 mM colchicine prior to incubation with BoNT B for 90 min. In the presence of colchicine, the samples were washed and NA release quantified. The continued presence of colchicine reduced the Ca^{2+}-dependent K^+-evoked release of NA from 8.3±1.2 to 4.5±0.1% of the total content. Results are the mean (± S.D.) for 3 experiments. In (B), synaptosomes were pretreated at 37°C with 10 μM taxol for 60 min, then simultaneously with 1 mM colchicine for an additional 30 min before simultaneous exposure to BoNT B for 90 min. The synaptosomes were then washed and release measured in the presence of 10 μM taxol (▲); controls were treated with colchicine alone (●) or BoNT B alone (■). The number of independent experiments for each value is indicated by the value shown, and the significance of differrence between taxol/colchicine-treated synaptosomes and those treated with colchicine alone is indicated by asterisks above the paired points (*, 10%). Note that the presence of taxol had no measurable effect upon Ca^{2+}-dependent K^+-evoked NA release. From Dolly et al. (1991).

Fig. 21. Selective phosphorylation of a large protein from nerve terminals after intoxication with BoNT. Rat cerebrocortical synaptosomes were incubated with [^{32}P]-Pi and either with 360 nM BoNT B (A) or without toxin (B) for 90 min at 37°C. After washing, the samples were exposed to 1.2 mM Ca^{2+} for 30s prior to cessation of phosphorylation by the addition of a cocktail of inhibitors of kinases/phosphatases. Samples were then prepared for and analysed by 2D-PAGE employing 3-10% gradient gels in the second dimension. This figure represents the resultant autoradiograms prepared from the 2D gels. The relevant pH range is indicated on horizontal axis, and the position of M_r protein standards are indicated by lines on the vertical axis. These markers in ascending order [M_r (kDa)] were: 20, soybean trypsin inhibitor; 24, trypsinogen; 29, carbonic anhydrase; 36, glyceraldehyde-3-phosphate dehydrogenase; 45, ovalbu-

min; 66, bovine serum albumin; 97, phosphorylase b; 116, β-galactosidase; 160, macroglobulin. The position of the BoNT sensitive protein is indicated by an arrowhead (M_r ≈180 kDa) and arrows (pI ≈5.0).

Table. 1. Biochemical properties of ^{125}I-BoNT A binding sites in detergent solubilised rat diaphragm and cerebrocortical synaptosomes

The innervated region of rat diaphragm or preparations of rat cerebrocortical synaptosomal membranes were extracted with detergent, immobilized onto nitrocellulose discs, and incubated (for 1h at 22 C in Krebs bicarbonate buffer, pH 7.4 containing 1 mg/ml BSA) with 1 nM ^{125}I-BoNT A in the presence or absence of 250 nM of the unlabelled neurotoxin or fragments shown below. The values of saturable binding presented are the mean of triplicate determinations SD; a qualitatively similar pattern of binding was seen with two preparations of tissue extracts and toxin samples.

Tissue	Saturable ^{125}I-BoNT A binding (fmol/mg/protein)	
	Innervated diaphragm	Synaptosomal membranes
Untreated tissue	98±11	88±3
Trypsinised*	0.2±0.2	0
Incubated with neuraminidase*	94±6	0
1 nM ^{125}I-BoNT A alone	155±8	91±3
+ 250 nM: BoNT A	53±4	29±5
: HC	154±4	31±4
: H_2L	148±18	98±12
: H_2	184±4	84±6

*A portion of innervated rat diaphragm and an aliquot of synaptosomes were incubated at 37 C for 1h with 1 mg/ml of trypsin or 1 unit/ml of neuraminidase prior to detergent extraction.

List of all personnel who received pay (in some cases for part of the duration) from the contract

C. McInnes
S. Lande
I. Matsuoka
D. Parcej
A. Ashton
J. Wadsworth
G. Tibbs
J.O. Dolly

Graduate degrees arising from contract support

J. Wadsworth, Ph.D.) supported indirectly
A. Maisey, Ph.D.)

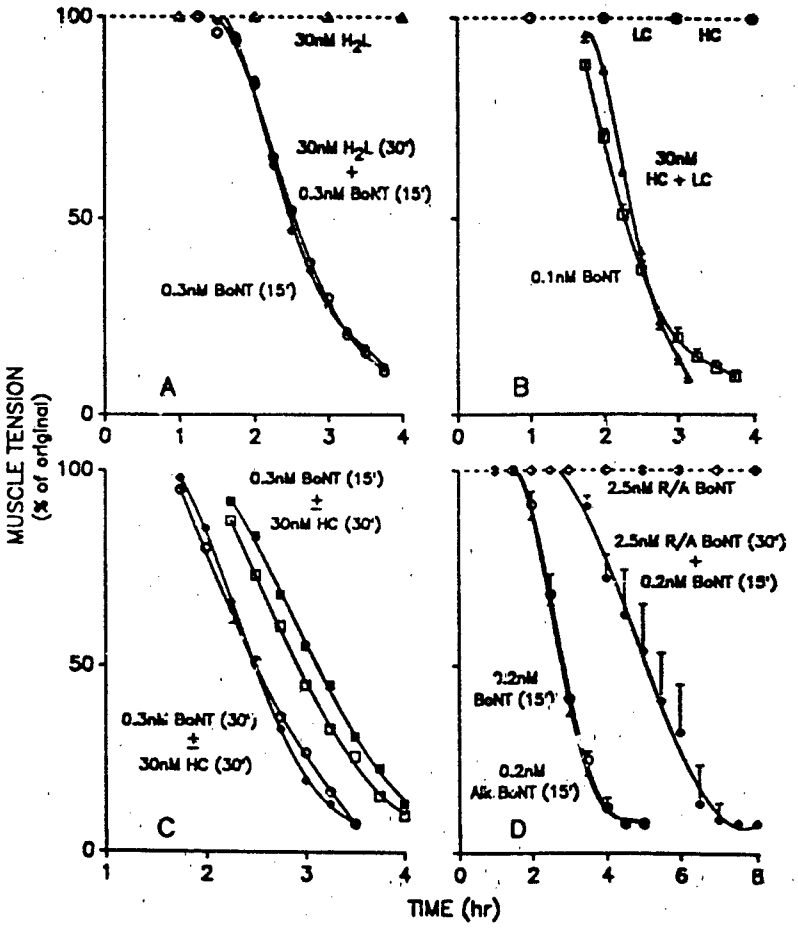
MUSCLE TENSION
(% of original)
TIME (hr)
30nM H_2L
30nM H_2L (30')
+
0.3nM BoNT (15')
0.3nM BoNT (15')
A
LC
HC
30nM
HC + LC
0.1nM BoNT
B
0.3nM BoNT (15')
±
30nM HC (30')
0.3nM BoNT (30')
±
30nM HC (30')
C
2.5nM R/A BoNT
2.5nM R/A BoNT (30')
+
0.2nM BoNT (15')
2.2nM
BoNT (15')
0.2nM
Alk. BoNT (15')
D

FIGURE 1

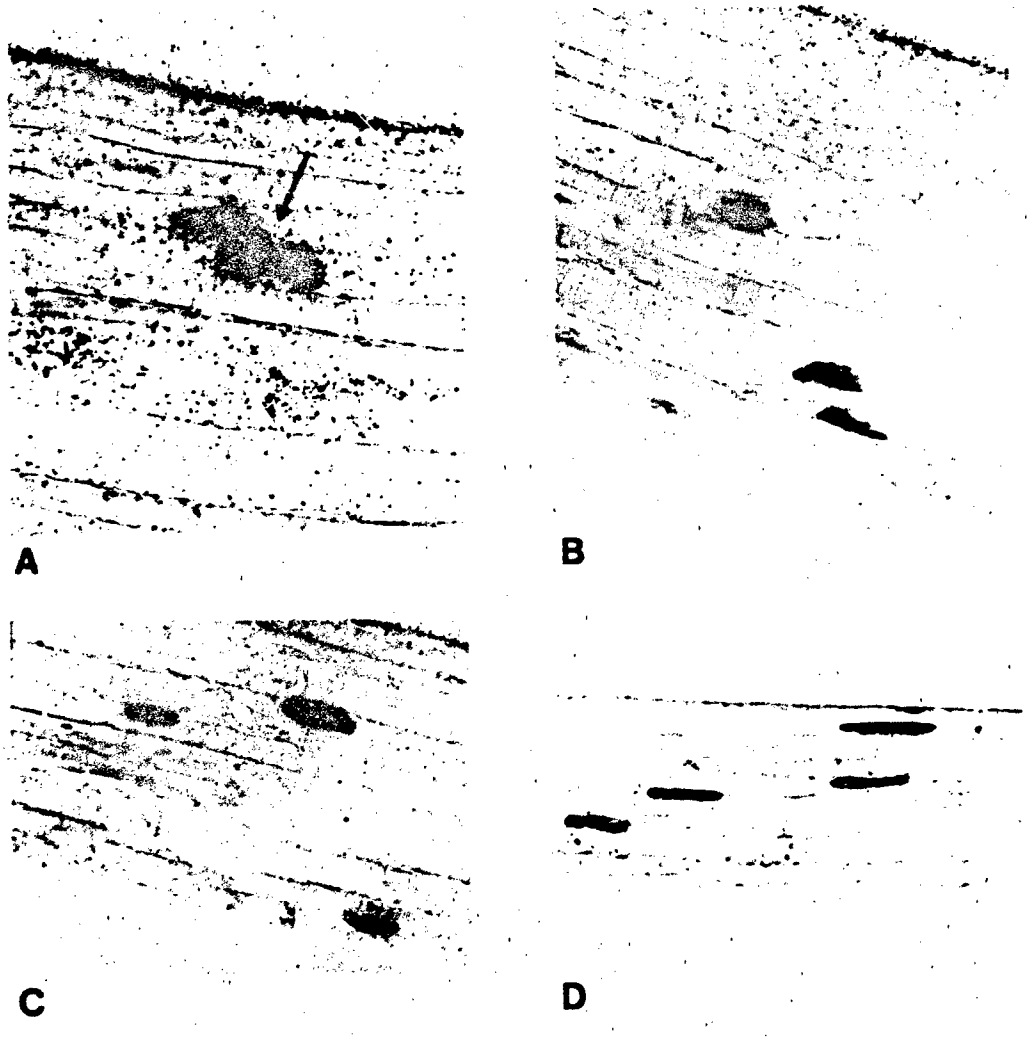

FIGURE 2

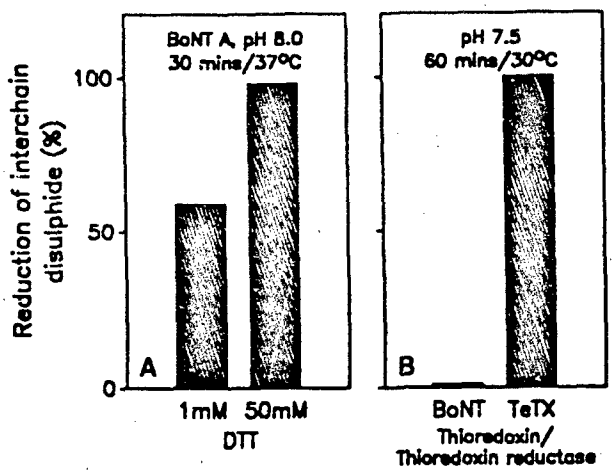
BoNT A, pH 8.0
30 mins/37°C
pH 7.5
60 mins/30°C
Reduction of interchain disulphide (%)
100
50
0
A
B
1mM 50mM
DTT
BoNT TeTX
Thioredoxin/
Thioredoxin reductase

FIGURE 3

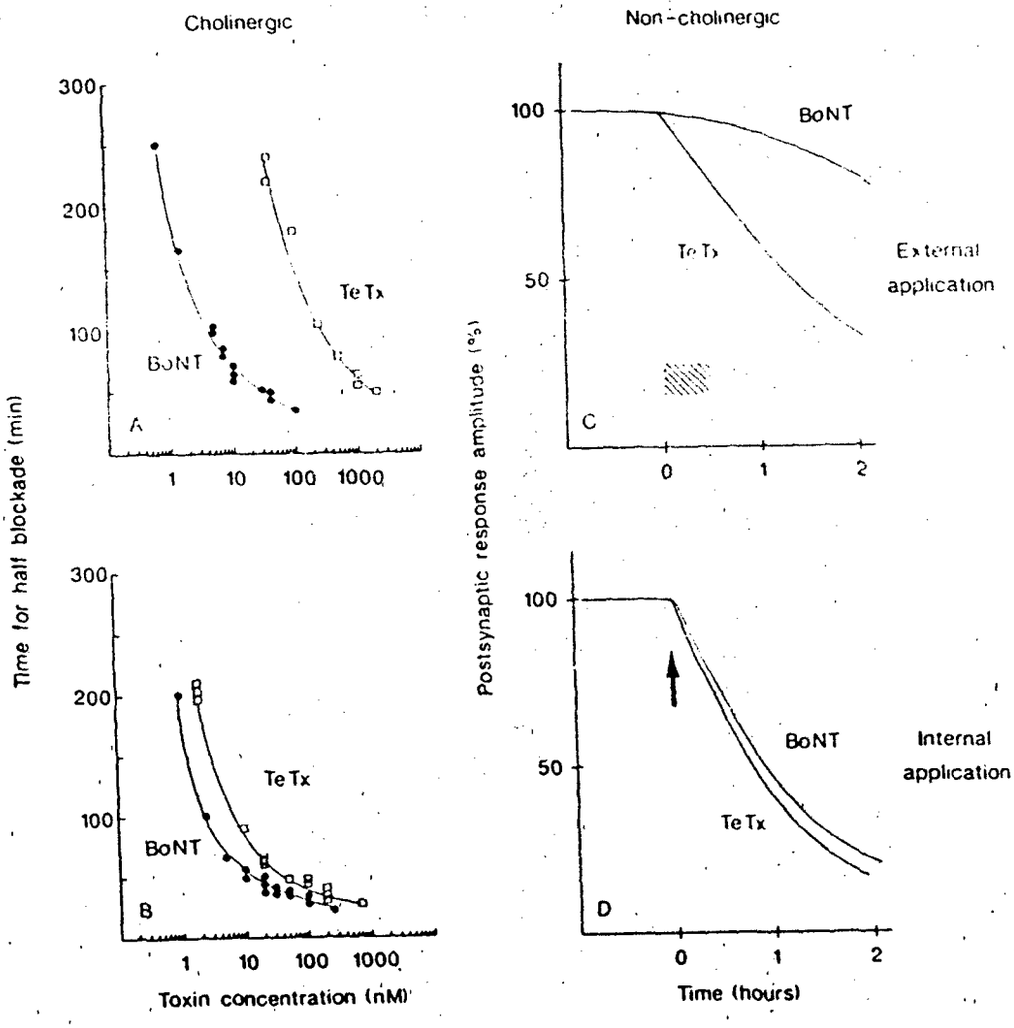
Cholinergic
Non-cholinergic
Time for half blockade (min)
300
200
100
TeTx
BoNT
A
1
10
100
1000
B
Toxin concentration (nM)
Postsynaptic response amplitude (%)
100
50
BoNT
TeTx
External application
C
0
1
2
Internal application
D
Time (hours)

FIGURE 4

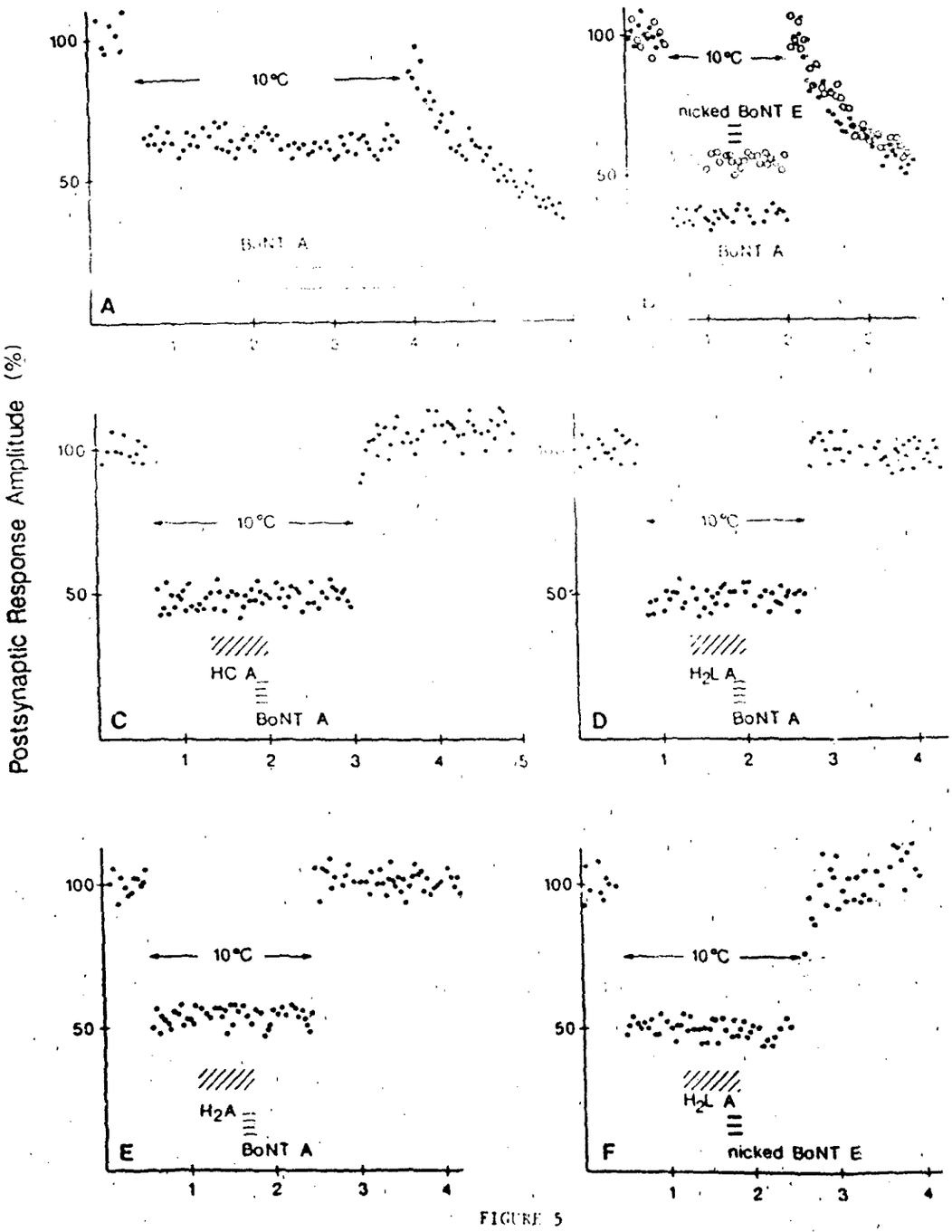
Postsynaptic Response Amplitude (%)
100
50
10°C
BoNT A
A
nicked BoNT E
HC A
H2L A
H2A
C
D
E
F
1
2
3
4
5
Time (hours)

FIGURE 5

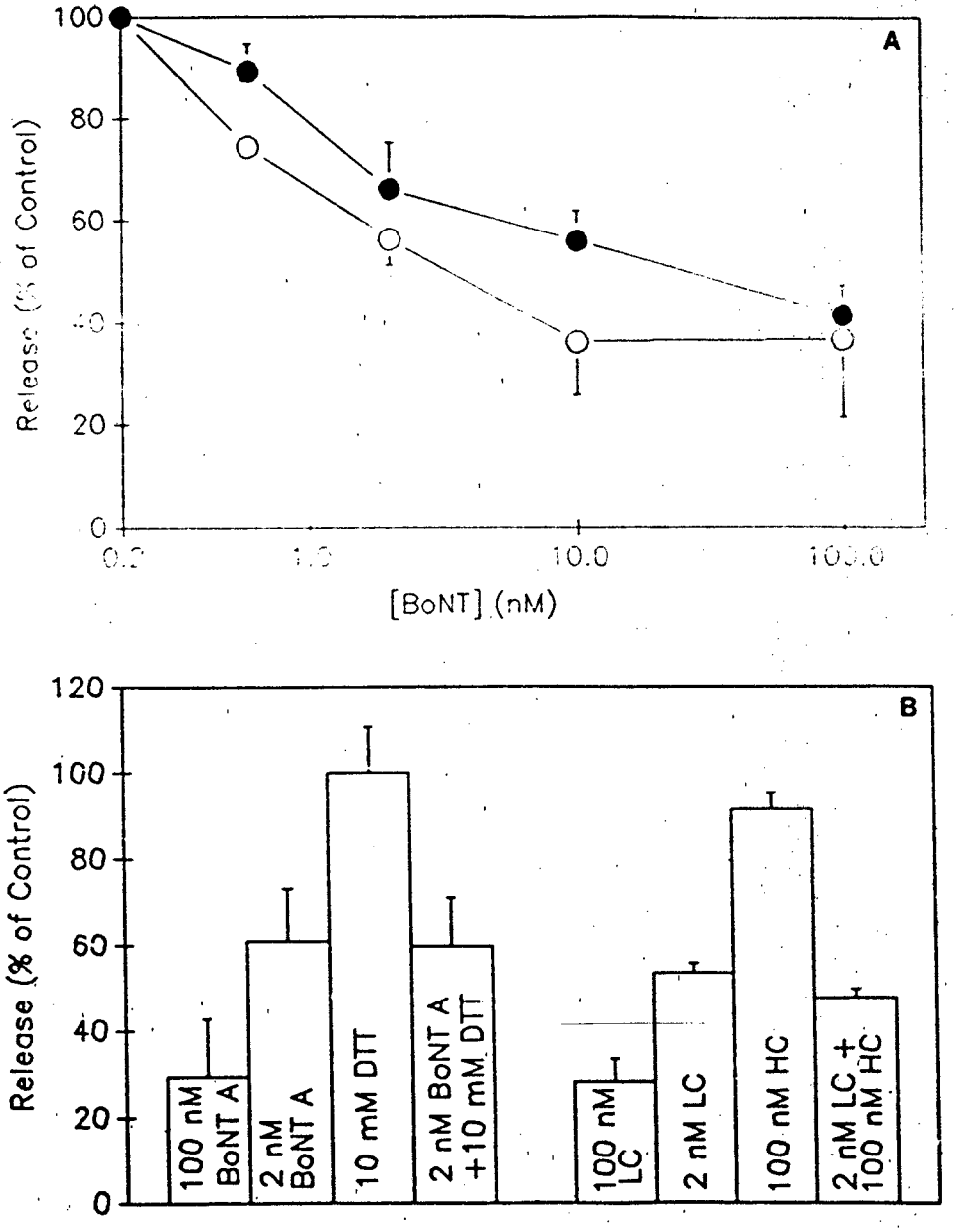
A
100
80
60
40
20
0
Release (% of Control)
0.2
1.0
10.0
100.0
[BoNT] (nM)
B
120
100
80
60
40
20
0
Release (% of Control)
100 nM BoNT A
2 nM BoNT A
10 mM DTT
2 nM BoNT A +10 mM DTT
100 nM LC
2 nM LC
100 nM HC
2 nM LC + 100 nM HC

FIGURE 6

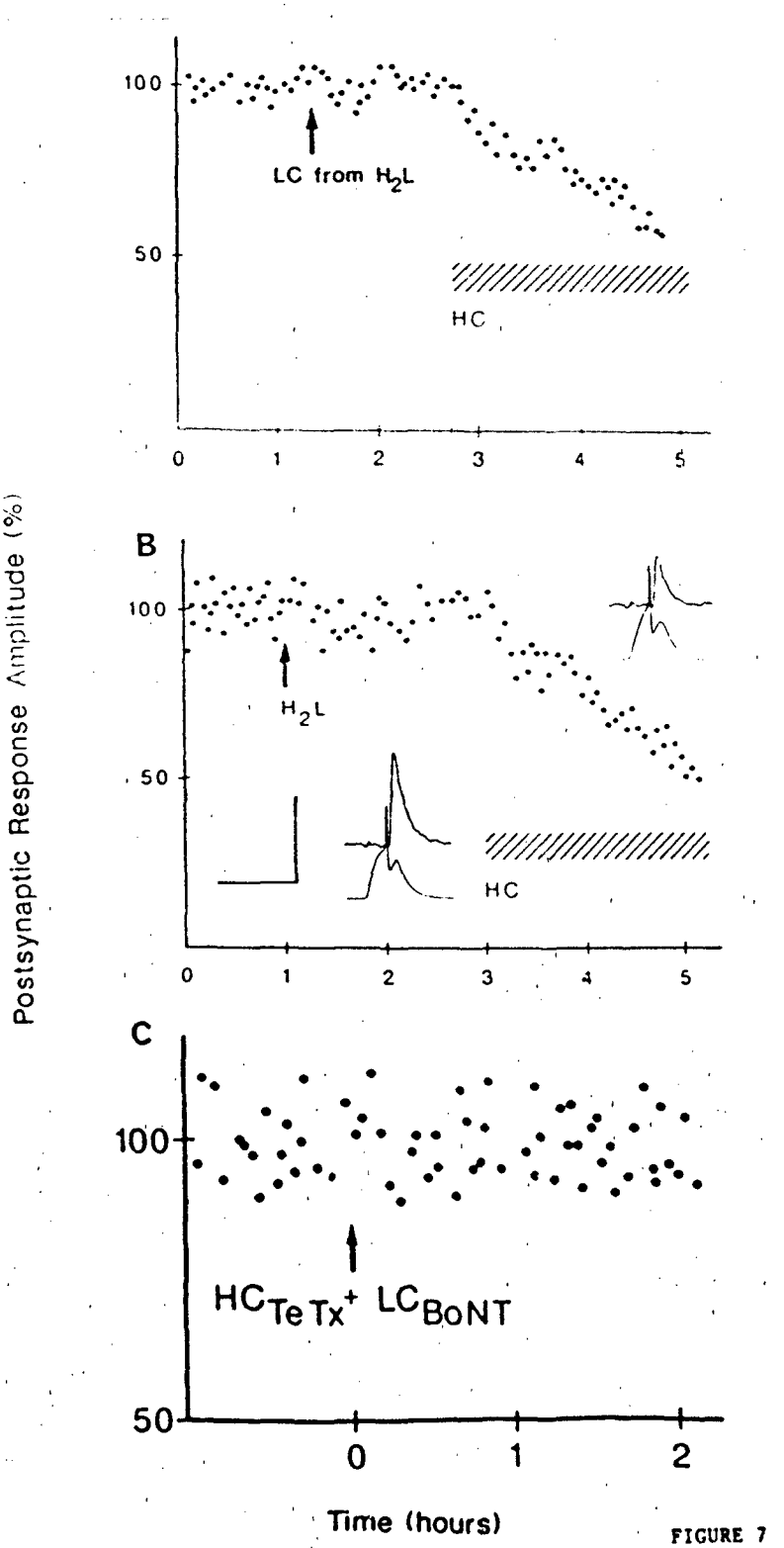
100
50
LC from H_2L
HC
0
1
2
3
4
5
B
100
50
H_2L
HC
0
1
2
3
4
5
C
100
50
HC_{TeTx} + LC_{BoNT}
0
1
2
Time (hours)
Postsynaptic Response Amplitude (%)

FIGURE 7

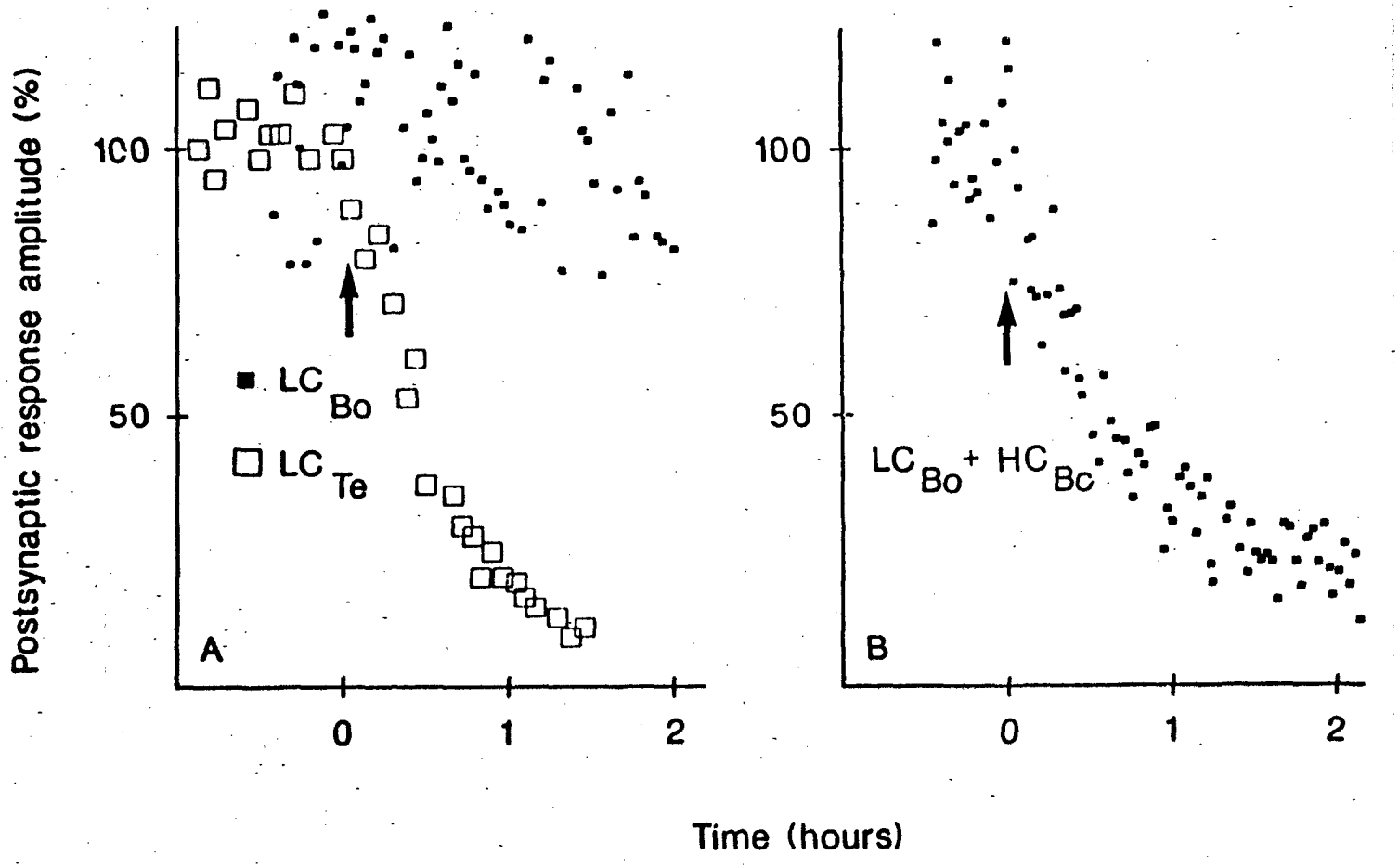
Postsynaptic response amplitude (%)
100
50
LC Bo
LC Te
A
0
1
2
100
50
LC Bo + HC Bc
B
0
1
2
Time (hours)

FIGURE 8

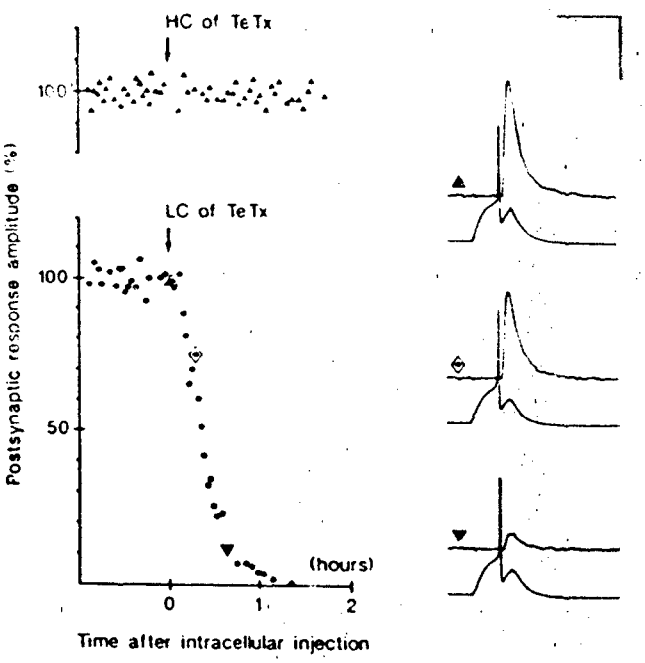
HC of TeTx
100
LC of TeTx
100
50
Postsynaptic response amplitude (%)
(hours)
0
1
2
Time after intracellular injection

FIGURE 9

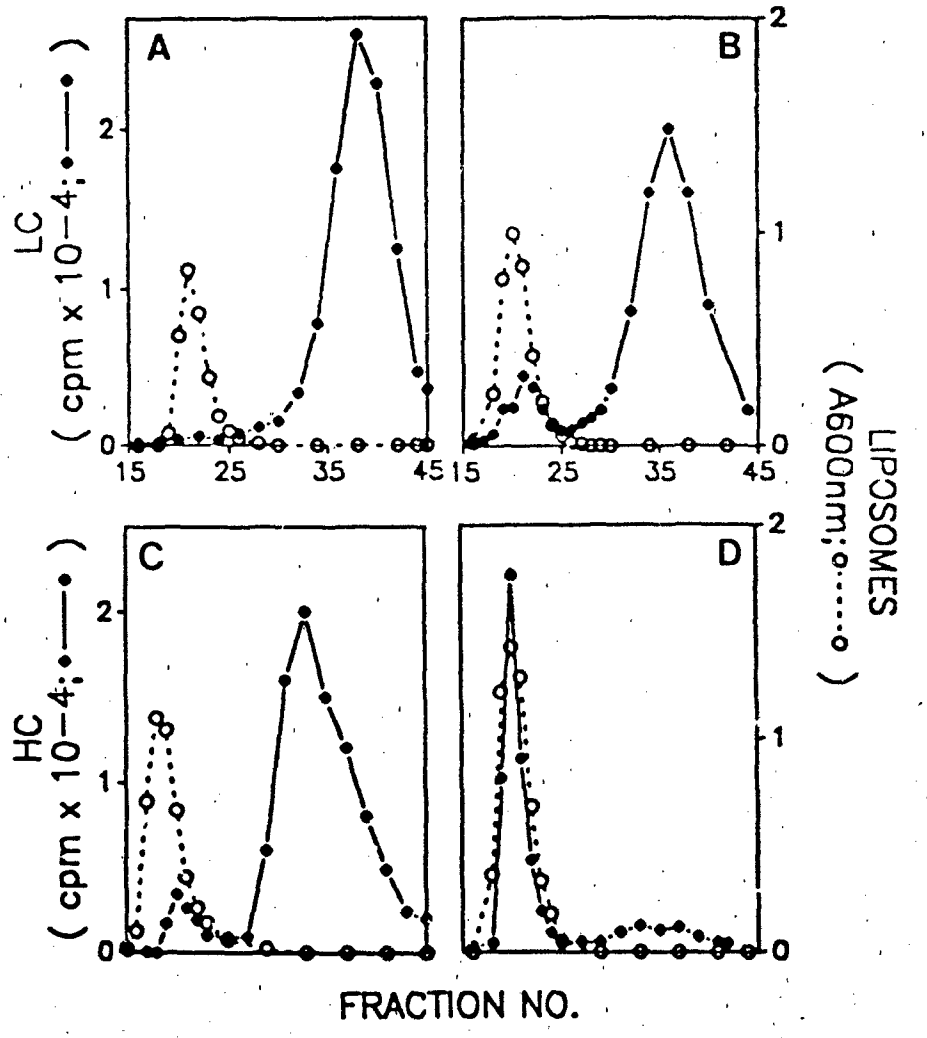
A
B
C
D
LC
(cpm x 10−4; •——•)
HC
(cpm x 10−4; •——•)
LIPOSOMES
(A600nm; o·····o)
FRACTION NO.
15
25
35
45
0
1
2

FIGURE 10

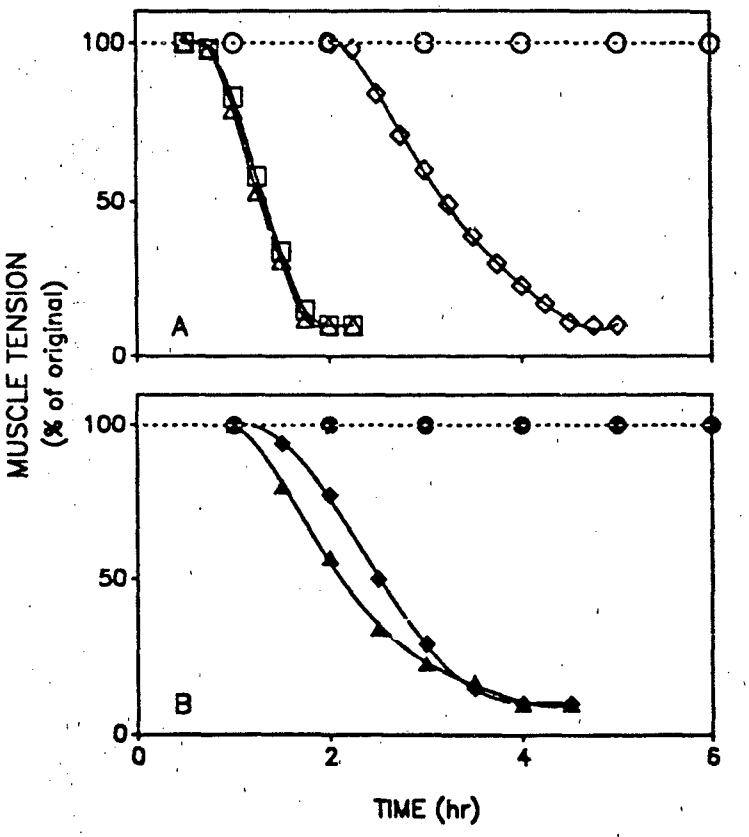

FIGURE 11

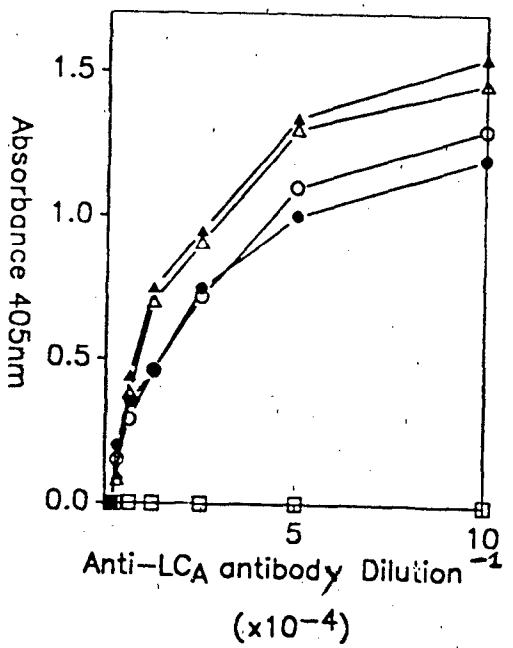
1.5
1.0
0.5
0.0
Absorbance 405nm
5
10
−1
Anti−LCA antibody Dilution
(x10−4)

FIGURE 12

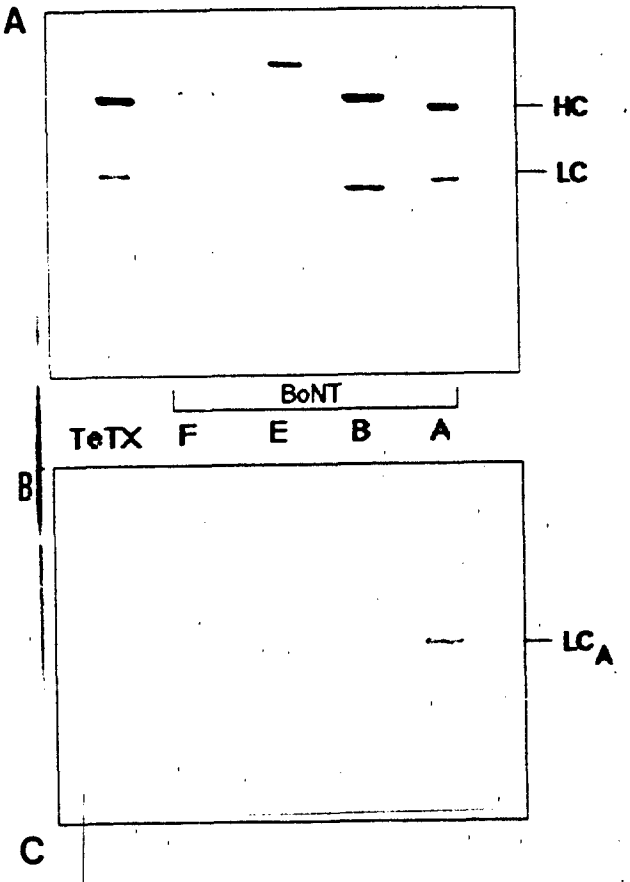

FIGURE 13

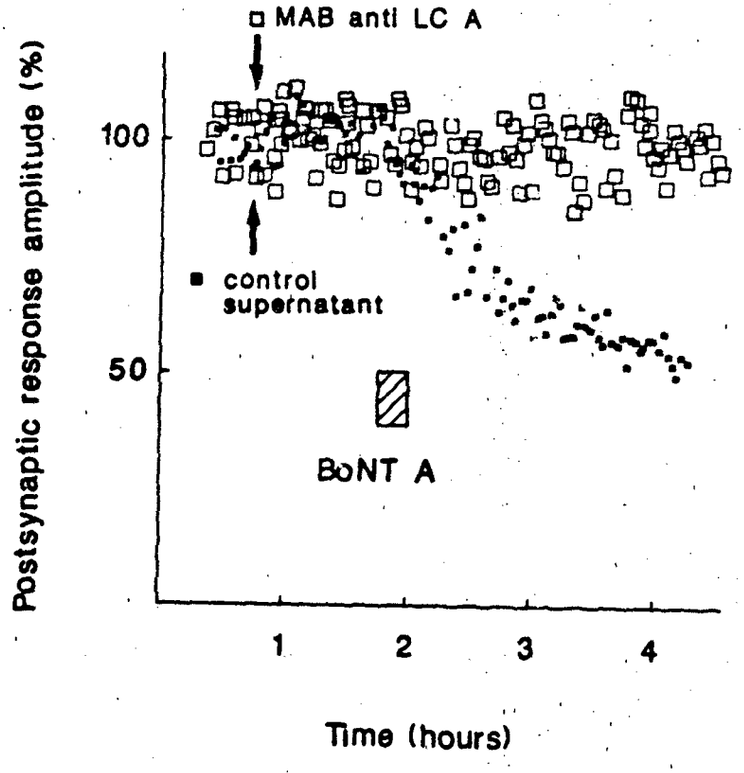
□ MAB anti LC A
■ control supernatant
BoNT A
Postsynaptic response amplitude (%)
100
50
1
2
3
4
Time (hours)

FIGURE 14

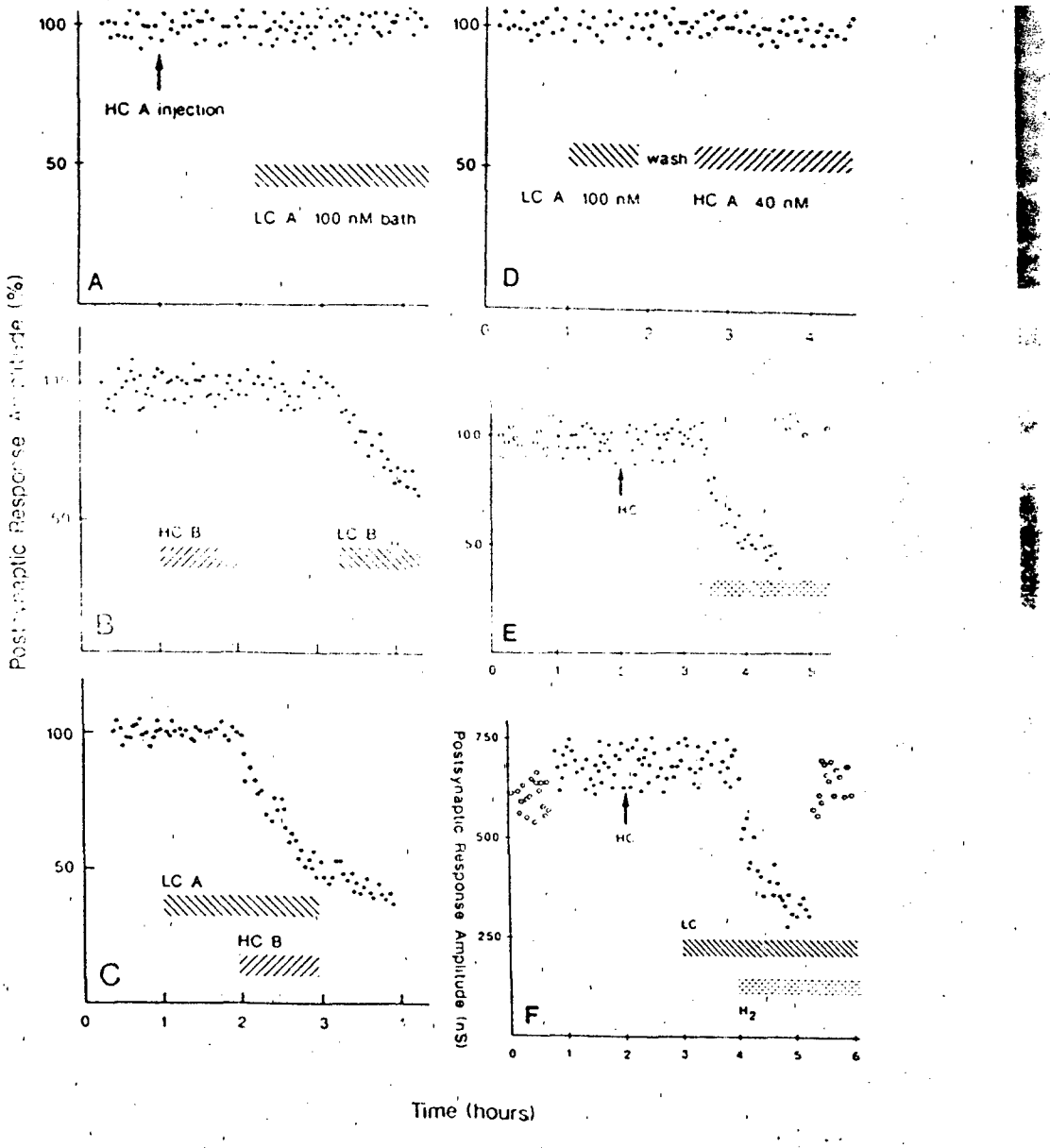
Postsynaptic Response Amplitude (%)
100
50
HC A injection
LC A 100 nM bath
A
wash
LC A 100 nM
HC A 40 nM
D
HC B
LC B
B
HC
E
LC A
HC B
C
Postsynaptic Response Amplitude (nS)
750
500
250
HC
LC
H_2
F
Time (hours)

FIGURE 15

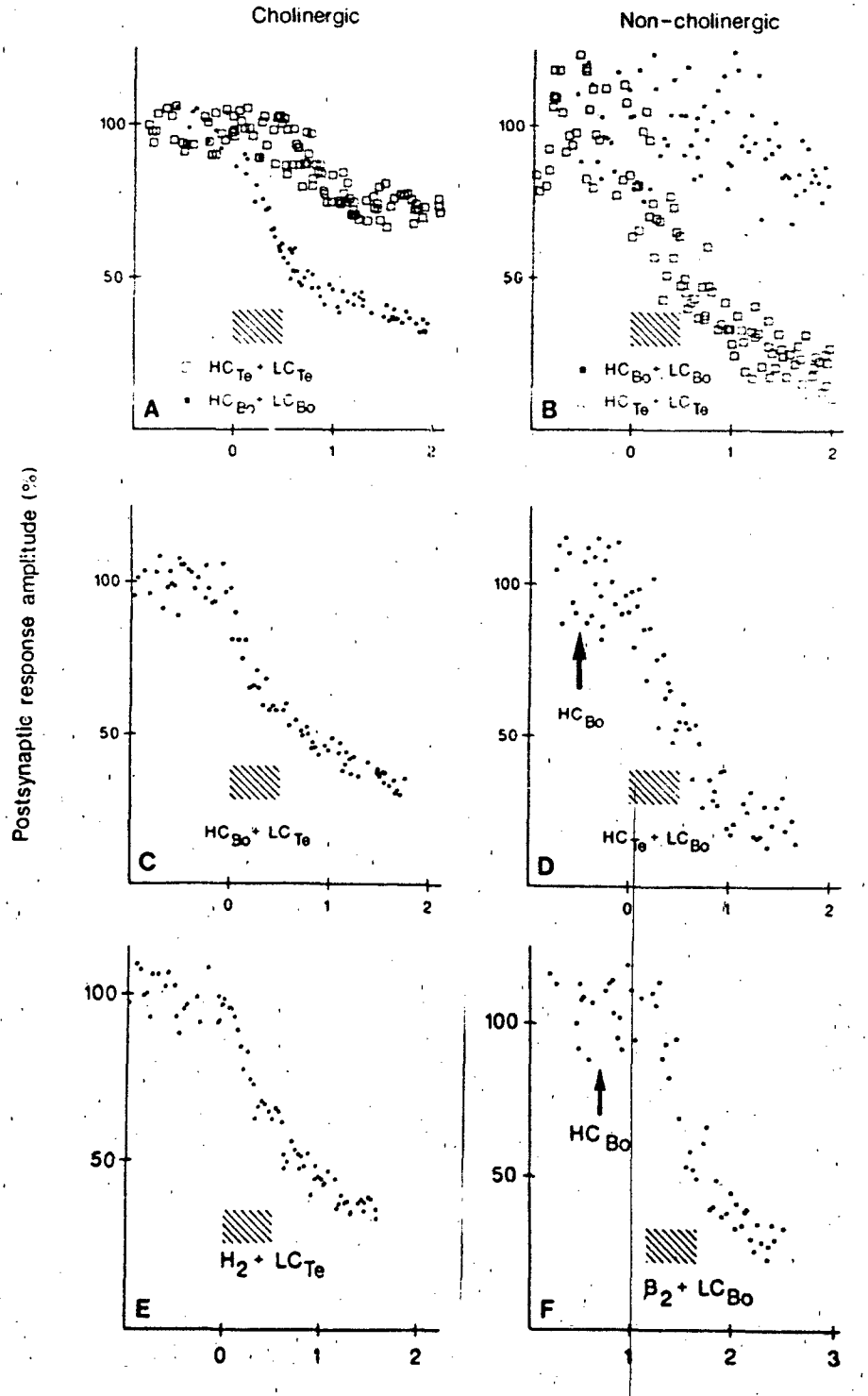
Cholinergic
Non-cholinergic
Postsynaptic response amplitude (%)
100
50
A
HC_{Te} + LC_{Te}
HC_{Bo} + LC_{Bo}
B
HC_{Bo} + LC_{Bo}
HC_{Te} + LC_{Te}
C
HC_{Bo} + LC_{Te}
D
HC_{Bo}
HC_{Te} + LC_{Bo}
E
H_2 + LC_{Te}
F
HC_{Bo}
β_2 + LC_{Bo}
0
1
2
3
Time (hours)

FIGURE 16

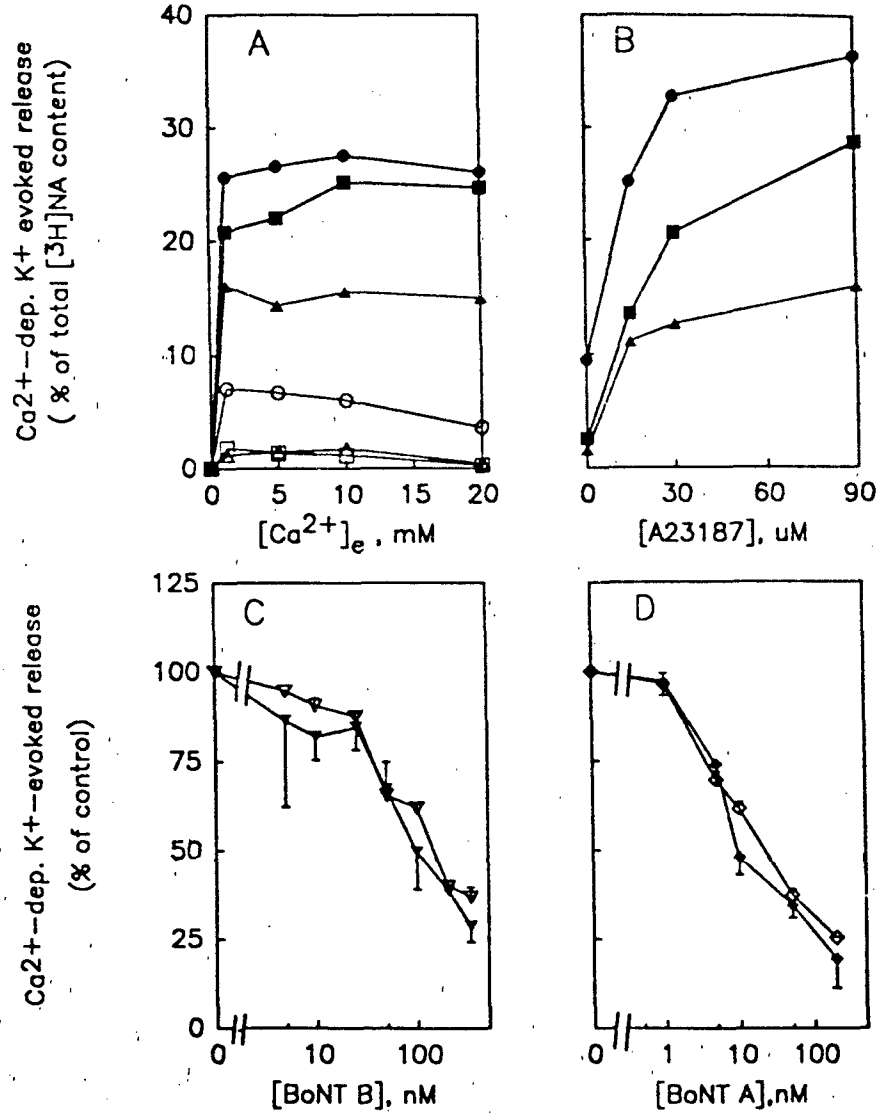
Ca2+-dep. K+ evoked release
(% of total [3H]NA content)
A
B
40
30
20
10
0
0 5 10 15 20
[Ca2+]e , mM
0 30 60 90
[A23187], uM
Ca2+-dep. K+-evoked release
(% of control)
C
D
125
100
75
50
25
0
0 10 100
[BoNT B], nM
0 1 10 100
[BoNT A],nM

FIGURE 17

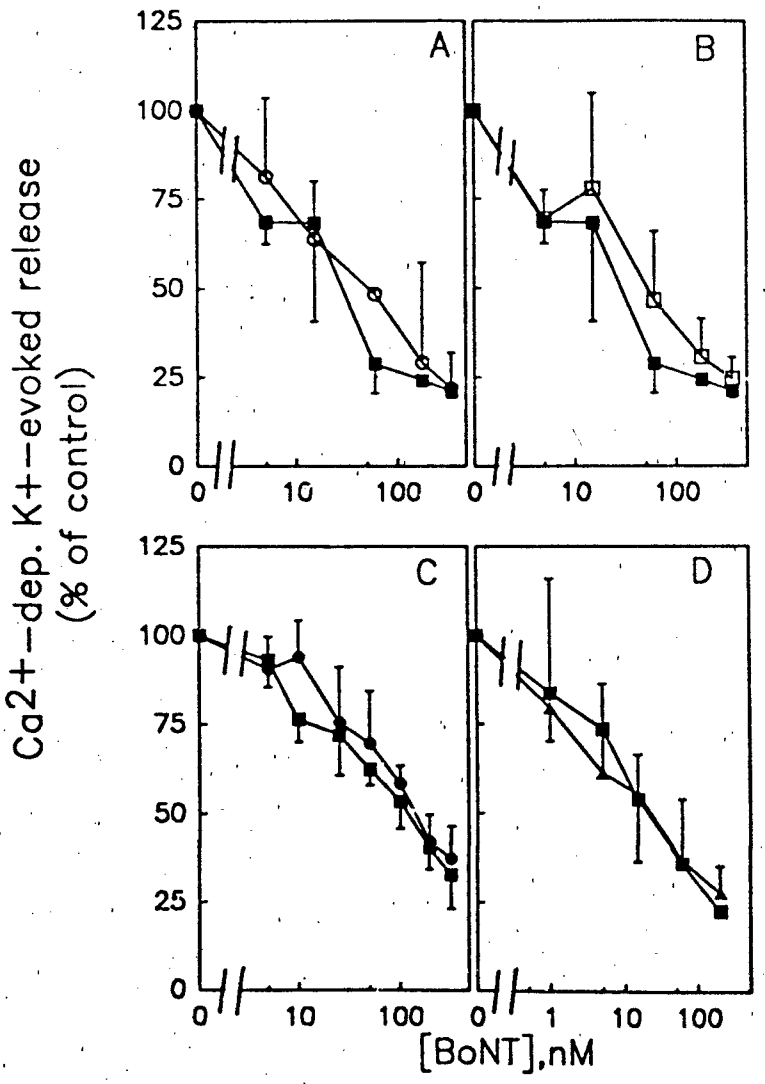
Ca2+-dep. K+-evoked release
(% of control)
125
100
75
50
25
0
A
B
C
D
0
10
100
1
[BoNT],nM

FIGURE 18

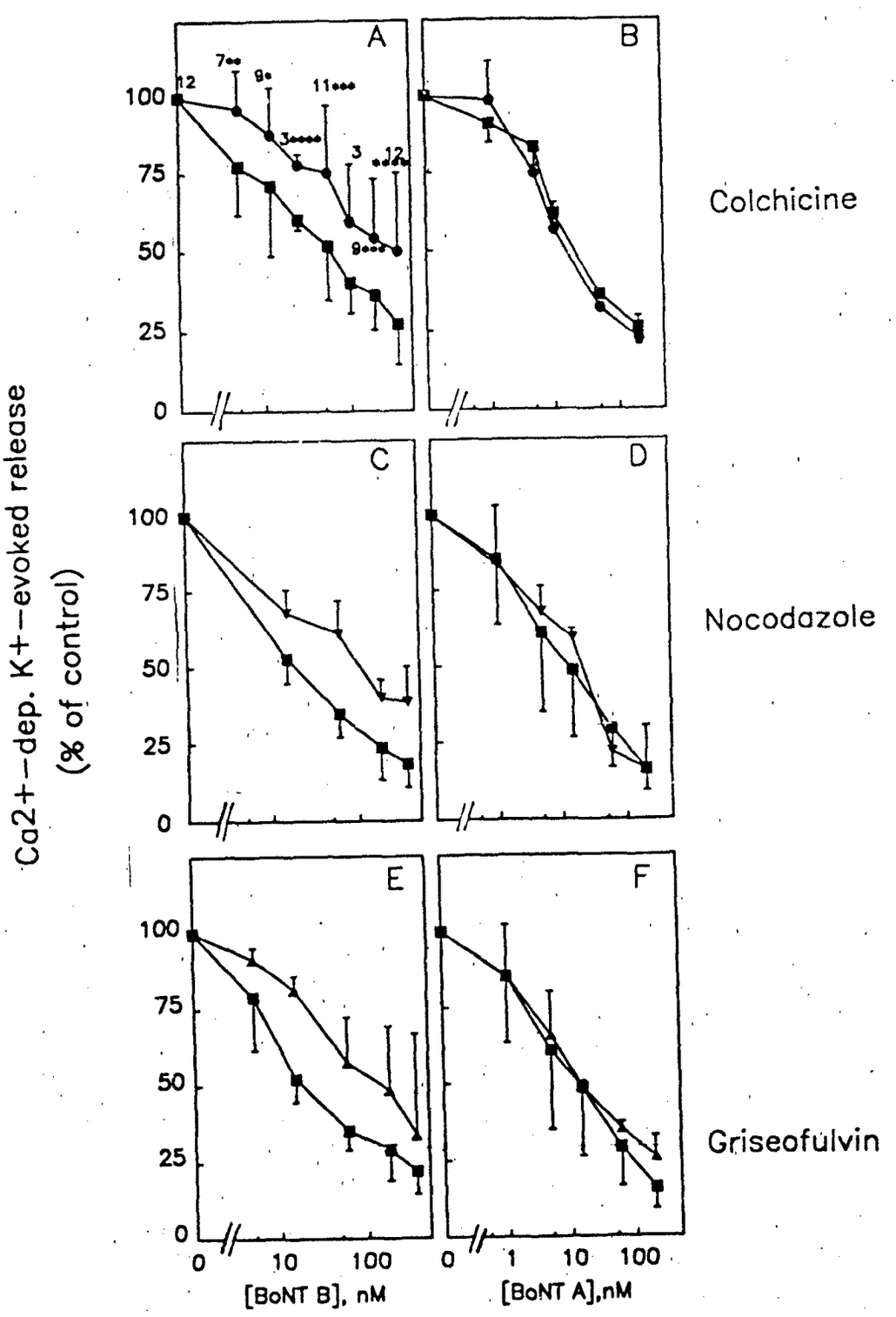
A
B
C
D
E
F
Colchicine
Nocodazole
Griseofulvin
Ca2+-dep. K+-evoked release
(% of control)
100
75
50
25
0
12
7**
9*
11***
3****
3
12
9***
0
10
100
1
[BoNT B], nM
[BoNT A],nM

FIGURE 19

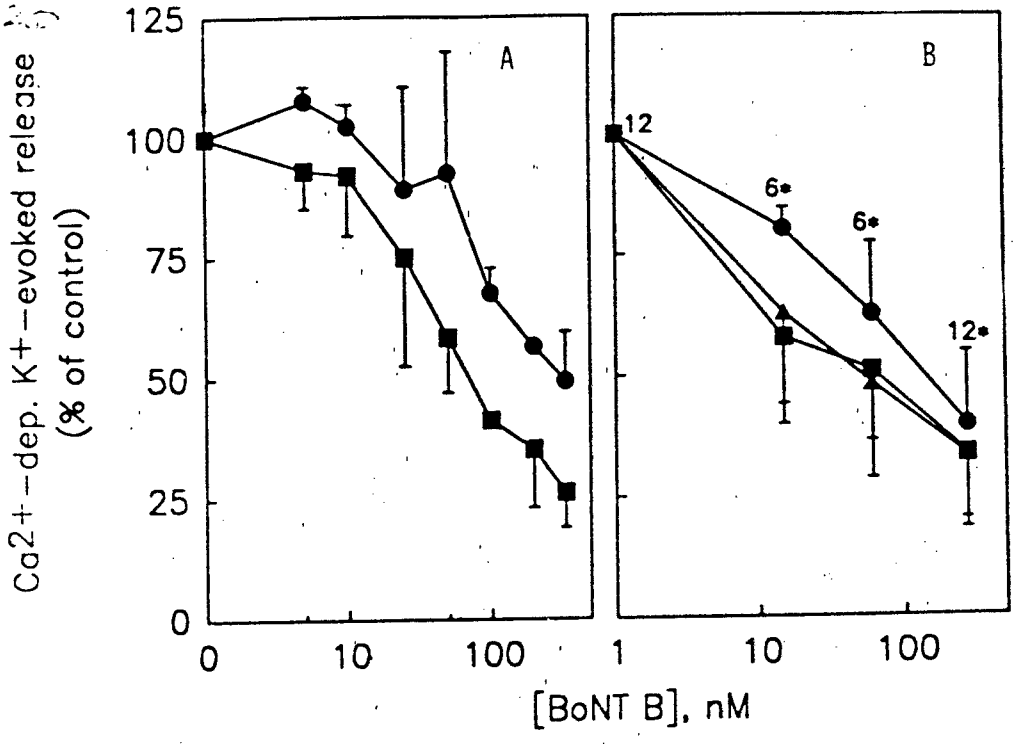
Ca2+-dep. K+-evoked release
(% of control)
125
100
75
50
25
0
A
B
12
6*
6*
12*
0
10
100
1
10
100
[BoNT B], nM

FIGURE 20

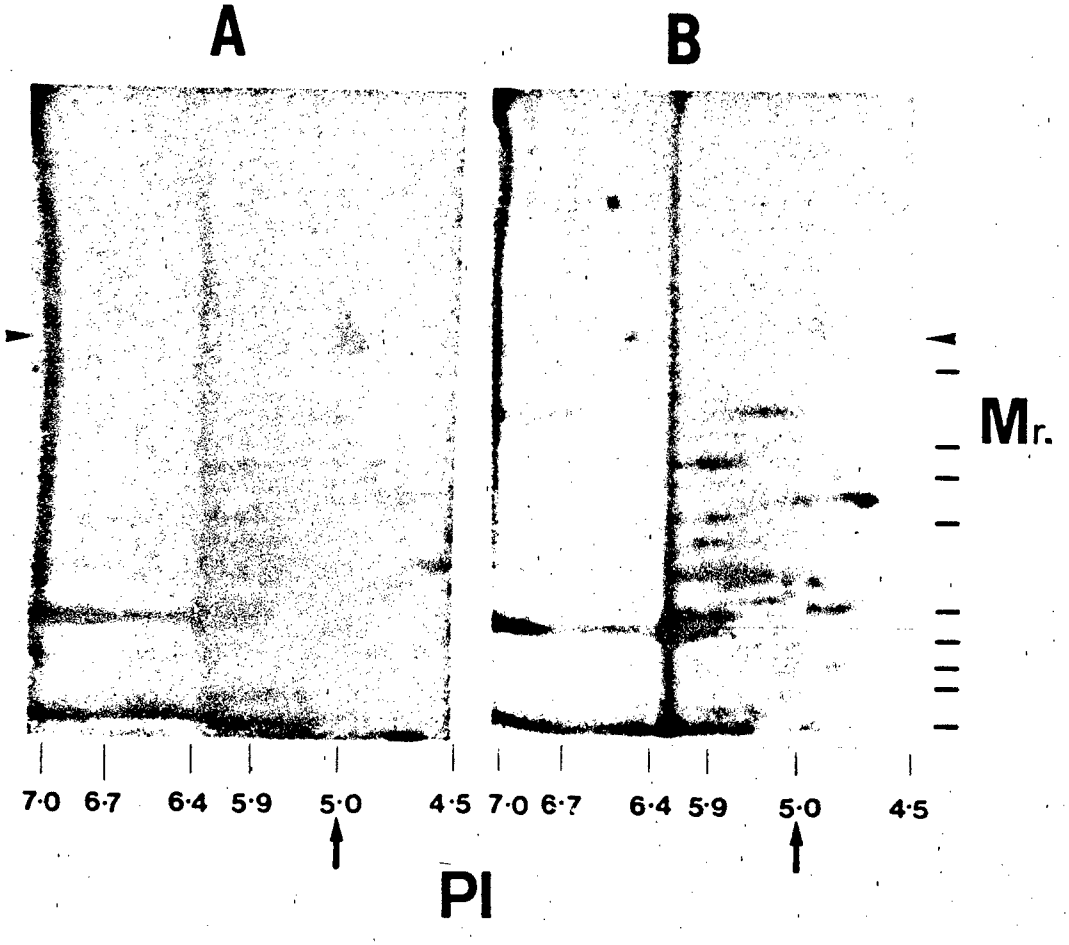

FIGURE 21